IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No:	10/577,840
Applicant:	Pere Joan Cardona Iglesias *et al.*
Filed:	February 1, 2007
Title:	Immunotherapeutic agent for the combined treatment of tuberculosis in association with other drugs
TC/A.U.:	1633
Examiner:	Fereydoun Ghotb Sajjadi
Docket No.:	TJA-139US

DECLARATION UNDER 37 C.F.R. §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir,

I, Pere-Joan Cardona, hereby declare and state that:

I am a citizen of Spain;

I have received a medical degree from the Universitat Autonoma de Barcelona;

I am Head of the Experimental Tuberculosis Unit of Germans Trias i Pujol Health Sciences Research Institute;

I am the author of several publications related to tuberculosis, listed in the Curriculum vitae attached hereto, including articles related to a new hypothesis for explaining the latent infection caused by Mycobacterium tuberculosis;

I am one of the inventors of the above mentioned patent application;

I have read the Office Action dated August 7, 2009 and understand the basis of the rejections set forth therein and the references cited by the Examiner, in particular Andersen *et al.* and Chaturvedi *et al.*; and that

Neither Andersen *et al.* nor Chaturvedi *et al.* disclose a method for preparing cell wall fragments of mycobacteria comprising a step of homogenizing the cells with a non-ionic surfactant such as Triton X-114.

It is apparent to me and, in my opinion, would be to any skilled in the art of microbiology and tuberculosis that a process for preparing cell wall fragments from Mycobacterium tuberculosis H37Rv strain by homogenization of the cells with a non-ionic surfactant selected from the group consisting of ethoxylated sorbitan esters and alkylphenol ethoxylates, such as Triton® X-114, followed by isolation of the insoluble cell wall fragments by centrifugation would render cell wall fragments with a different composition than if the cell wall fragments had been obtained by merely a step of sonication and centrifugation. Both processes will render complex mixtures and as such difficult to completely characterise. But since it is well known that the claimed non-ionic surfactants are able to solubilise lipids and some proteins, it is apparent that the homogenization in the presence of the non-ionic surfactant will solubilise at least part of the lipid compounds and proteins of the cell wall of the *M. tuberculosis*. Thus, such compositions will mainly differ in the lipidic content as well as in the way the remaining lipids and proteins are presented (e.g. the cell wall fragments obtained by the first method will lack the cell wall lipids and cell wall proteins solubilized by the surfactant treatment, proteins not solubilised would be more exposed devoid from most of surrounding lipids, and not solubilised lipids will be more finely divided by the emulsion formed during the homogenisation with the surfactant). Such differences in the composition are expected to result in different biological activities.

The biological activity of the immunotherapeutic agent does not depend on the lyophilization step.

The culture time period of at least 3 weeks, preferably from 3 to 4 weeks, is a feature which does result in structural differences. The relatively long claimed culturing time lead to the induction of microaerobic (or stressful) conditions similar to those that are thought to be found in granulomas that contain *Mycobacterium tuberculosis* latent bacilli, and therefore it may favour the production of antigens typical from these latent bacilli. In fact, when increasing the culturing time to at least three weeks, which is not optimal for achieving maximal cell density and viability (i.e., routine optimization parameters), a different antigenic protein profile was observed. These conditions would not be obtained with a lower culturing time such as those disclosed in Andersen *et al.* and Chaturvedi *et al.*

In my opinion, based on my knowledge and experience in the field of tuberculosis, at the time the instant invention was made one would not have had a reasonable expectation of succeeding in finding an immunotherapeutic agent against latent tuberculosis, based on a non-purified cell extract of a virulent tuberculosis strain, that would result in effectively reducing the number of bacilli and, also importantly, without inducing toxic responses, so that it could be potentially used to significantly reduce the currently employed long-term (of 6 to 9 months) chemotherapy treatment.

Studies published recently prior to the time the invention was made showed that even vaccines previously probed to be immunogenic when given to uninfected people, resulted ineffective when given as treatment of infected patients. Moreover, the art strongly

recommended not using post-infection vaccination with killed or alive BCG, as this was shown to induce strong tissue toxicity.

Thus, Moreira *et al.* (Mycobacterial antigens exacerbate disease manifestations in Mycobacterium tuberculosis-infected mice. Infect Immun 2002;70:2100–7) concluded that the reported "studies suggest that administration of mycobacterial antigens to mice with prior M. tuberculosis infection leads to immune activation that may exacerbate lung pathology via TNF-alpha-induced inflammation without reducing the bacillary load".

And Turner *et al.* (Effective preexposure tuberculosis vaccines fail to protect when they are given in an immunotherapeutic mode. Infect Immun 2000;68:1706–9) concluded that two tested DNA and subunit immunogenic vaccine formulations had no effect on the course of the infection in the lungs, though both reduced the bacterial load in the spleen, and inoculation with Mycobacterium bovis BCG not only had no effect but also when given more than once, appeared to induce an increasingly severe pyogranulomatous response in the lungs of the mice.

A DNA vaccine, encoding the 65-kDa heat shock protein of *Mycobacterium leprae* (hsp60/lep), initially designed to prevent infection, showed a pronounced therapeutic action (Lowrie *et al.* Therapy of tuberculosis in mice by DNA vaccination. Nature.1999 Jul 15;400(6741):269-71). However, Taylor *et al.* (Pulmonary Necrosis Resulting from DNA Vaccination against Tuberculosis. Infect Immun. 2003 April; 71(4): 2192–2198) reported later experiments concluding that:

> "this vaccine was not effective in a more realistic aerosol infection model or in a model of latent tuberculosis in the lungs. Moreover, when given in an immunotherapeutic model the immunized mice developed classical Koch reactions characterized by multifocal discrete regions of cellular necrosis throughout the lung granulomas."

The use of *M. vaccae* as an adjuvant for the treatment of tuberculosis together with administration of rifampizin and isoniazid has been suggested (cf. U.S. Patent No. 4,724,144). However, according to the Cochrane database (cf. Cochrane Database Syst Rev. 2003;(1):CD001166) it was of no benefit for patients, based on the results of several trials in humans. Thus, as concluded in the abstract:

> "There was no effect on mortality (4 trials, OR 1.09, 95% CI 0.79 to 1.49). No consistent effect on sputum negativity or sputum culture was shown. Most immunotherapy recipients experienced local adverse reactions (2 trials, OR 18.2, 95% CI 9 to 37), some of which progressed to ulceration and scarring. REVIEWER'S CONCLUSIONS: *Mycobacterium vaccae* does not benefit patients with tuberculosis."

The examples provided in the application, in particular, example 3 (IV) trial 4, show that the immunotherapeutic agent claimed in the instant invention greatly reduces the number of total bacilli including the number of latent bacilli in aerosol infected mice previously treated with short term chemotherapy. Thus, after a short chemotherapy treatment of only 8 weeks e.g. with isoniazid and rifampicin, only three doses of a composition of the immunotherapeutic agent instantly claimed allow to extremely reduce the number of bacilli in the lungs in a murine model of chronical tuberculosis infection, and, also importantly, without inducing local or systemic toxicity. It is also to be highlighted that the immunotherapeutic activity was observed without the need of additional adjuvants.

In fact, RUTI, a vaccine obtained by the process claimed in the present patent application, is the first therapeutic vaccine for the treatment of latent tuberculosis based on a non-purified mixture of antigens to reach phase I clinical trials.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 02th february 2010 .

Pere-Joan Cardona

CURRICULUM VITAE

PERSONAL DETAILS

NAME: Pere Joan Cardona Iglesias
DNI: 39341435G
BIRTH DATE: 14/04/1967
ADDRESS: C/ Osona nº 3, àtic 1ª 08023 Barcelona.
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ACADEMIC DETAILS

1992	Medical Degree (Universitat Autònoma de Barcelona).
1997	Specialisation degree for MD in Microbiology and Parasitology (Universitat Autònoma de Barcelona).
1999	PhD in Medicine (Universitat Autònoma de Barcelona).
2006	Master in Science and Welfare of laboratory animals (Universitat Autònoma de Barcelona).
2009	Accreditation for the body of the Spanish University Professors by ANECA (National Agency for the Evaluation of Quality)

Professional Experience

1990-1992	Intern fellowship (Lab. Mod. Infección. H. Vall d'Hebron, Barcelona, Spain).
1992-1993	Fellowship in the PhD program of the Lab. Mod. Infección. H. Vall d'Hebron (Barcelona, Spain).
1994-1997	Specialisation MIR in Microbiology and Parasitology (Hospital Universitari Germans Trias i Pujol, Badalona, Spain).
1998-1999	PhD student in the Experimental Tuberculosis Unit of the Hospital Universitari Germans Trias i Pujol (Badalona, Spain).
2000-2001	Researcher. Experimental Tuberculosis Unit of the Hospital Universitari Germans Trias i Pujol (Badalona, Spain).
2001-today	Associate Professor (Universitat Autònoma de Barcelona).
2002-2008	Researcher FIS. Fundació Institut Germans Trias i Pujol (Badalona, Spain).

Thesis directed

2003	PhD student: Jordi Torrelles Bertran Title: Study of Cell Wall Components in Mycobacterium spp. Their Implications in Pathogenesis and Epidemiology University: Universitat Autònoma Barcelona Qualification: Excellent cum laude
2004	PhD student: Sergi Gordillo Muñoz Title: Estudi de la latència de Mycobacterium tuberculosis en models experimentals de ratolí I "in vitro" University: Universitat Autònoma Barcelona Qualification: Excellent cum laude
2008	PhD student: Evelina Guirado Cáceres Title: Caracterització de la resposta immune induída per RUTI en el tractament de la infecció tuberculosa latent experimental University: Universitat Autònoma Barcelona Qualification: Excellent cum laude
2009	PhD student: Cristina Vilaplana Massaguer Title: Avaluació de la immunogenicitat de la vacuna RUTI® en humans University: Universitat Autònoma Barcelona Qualification: Excellent cum laude

RESEARCH PROJECTS

FEDER 1999. IP: Vicenç Ausina Ruiz.
"Estudio de una nueva pauta de corta duración de antibióticos antituberculosos en un modelo murino experimental basada en el uso de Nitroimidazoles y la encapsulación de los fármacos en liposomas".

FIS 99/0049-02. IP: Vicenç Ausina Ruiz.
"Estudio, en un modelo experimental murino, de los distintos grados de virulencia de las cepas de Mycobacterium tuberculosis resistentes a la isoniazida y su correlación con la composición glicolipídica de la pared celular, el tipo de respuesta inflamatoria pulmonar y las bases genéticas responsables".

SEPAR 2000. IP: Joan Lonca Jiménez.
"Estudio, en un modelo experimental murino, de los distintos grados de virulencia de las cepas de Mycobacterium tuberculosis resistentes a la isoniazida y su correlación con la composición glicolipídica de la pared celular, el tipo de respuesta inflamatoria pulmonar y las bases genéticas responsables".

FIS 01/0644 IP: Cardona Iglesias, Pere Joan.
"Caracterización de la expresión genética de *Mycobacterium tuberculosis* en fase de latencia en modelos experimentales en animales *in vitro*. Bases para la optimización del tratamiento de la tuberculosis".

SEPAR 2000. IP: Vicenç Ausina Ruiz.
"Quimioterapia de la tuberculosis. Estudio en un modelo experimental de una pauta esterilizante de corta duración basada en la vehiculización de fármacos antituberculosos en liposomas".

Collaboration contract with Archivel Technologies, S.L. 2000-2005.
IP: Cardona Iglesias, Pere Joan.
"Diseño de una vacuna contra la tuberculosis basada en antígenos estructurales en modelos de tuberculosis".

FIS 01/3104. IP: Cardona Iglesias, Pere Joan.
"Obtención de una vacuna para el tratamiento inmunoterápico de la infección por *Mycobacterium tuberculosis*".

FIS 03/0757. IP: Cardona Iglesias, Pere Joan.
"Evolución de las poblaciones de linfocitos T CD8 en el modelo experimental de tuberculosis en el ratón, de los clones más relevantes para el diseño de una vacuna sistemática".

SEPAR 2003. IP: Cardona Iglesias, Pere Joan.
"Diseño de una vacuna para el tratamiento inmunoterápico de la infección por *Mycobacterium tuberculosis*".

Proyecto SGR 2005 00056.GRUPO DE "MICROBIOLOGIA CLINICA Y PATOLOGIA INFECCIOSA EXPERIMENTALI (Grupo de Investigación de Cataluña acreditado por el Departament d'Universitats Recerca i Societat de la Informació, de la Generalitat de Catalunya). 2005-2008. IP: Vicenç Ausina Ruiz.

Collaboration contract with Archivel Farma S.L. 2006-2008.
IP: Cardona Iglesias, Pere Joan.
"Estudio de los mecanismos de protección generados por RUTI en el control de la infección tuberculosa latente".

Consortium Grand Challenge, Bill & Melinda Gates Foundation. 2005-2009. IP: Cardona Iglesias, Pere Joan.
" Preclinical and clinical evaluation of a post-exposure Tb vaccine".

Grant of the Ministerio de Educación y Ciencia. BIO002005-07949-C02-02. Periodo 2006-2008. IP: Cardona Iglesias, Pere Joan.
"Estudios de atenuación y protección en modelos de ratón, cobayo y cerdo de los candidatos a vacunas preventivas y terapéuticas basados en la inactivación de PHOP".

European Project: FP7- HEALTH-2007-2.3.2-2. 2008-2010.
IP: Cardona Iglesias, Pere Joan.
"LATENT TUBERCULOSIS: New tools for the detection and clearance of dormant *Mycobacterium tuberculosis*".

Proyecto PI080785 del Ministerio de Ciencia e Innovación. 2008-2011.
IP: Cardona Iglesias, Pere Joan.
"Demostración de la hipótesis dinámica de la infección tuberculosa latente"

Proyecto CB06/06/0031 del CIBER (Instituto de Salud Carlos III).ENFERMEDADES RESPIRATORIAS. Periodo 2007-2012. IP: Vicenç Ausina Ruiz.

European Project: FP7- HEALTH-2009-single-stage. 2010-2014
IP: Cardona Iglesias, Pere Joan.
"NEWTBVAC-Discovery and preclinical development of new generation tuberculosis vaccines".

European Project: FP7- SME-2008-1. 2010-2013
IP: Cardona Iglesias, Pere Joan.
"NOPERSIST-Novel strategies for the prevention and control of persistent infections".

OTHER ACHIEVEMENTS RELATED TO THE QUALITY OF THE RESEARCH PROJECTS

Ayuda a la investigación del Institut d'Estudis de la Salut. Novembre 1995. Project "Estudi de la resposta humoral contra antígens glicolipídics de la paret cel·lular de Mycobacterium tuberculosis en un model experimental d'infecció"

Evaluator of he research projects' proposals to the Ayudas del Programa de de Promoción de la Investigación Biomédica y en Ciencias de la Salud del Ministerio de Sanidad y Consumo de España, from 2003 until today.

PUBLICATIONS (SCIENTIFIC PAPERS) (Factor h= 15)

Internationals:

1996 Gavaldà J, Cardona PJ, Almirante B, Capdevila JA, Laguarda M, Pou L, Crespo E, Pigrau C, Pahissa A. "Treatment of experimental endocarditis due to Enterococcus faecalis using once-daily dosing regimen of gentamicin plus simulated profiles of ampicillin in human serum.". Antimicrob Agents Chemother. 1996 Jan;40(1):173-8.

1996 Giménez M, Sopena N, Viñado B, Cardona PJ, Pedro-Botet ML, Coroleu W, Arnal J. "Invasive Streptococcus agalactiae infections at a general university hospital over a 10-year period". Enferm Infecc Microbiol Clin. 1996 May;14(5):300-3.

1997 Gamboa F, Manterola JM, Viñado B, Matas L, Giménez M, Lonca J, Manzano JR, Rodrigo C, Cardona PJ, Padilla E, Dominguez J, Ausina V. "Direct detection of Mycobacterium tuberculosis complex in nonrespiratory specimens by Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test". J Clin Microbiol. 1997 Jan;35(1):307-10. Erratum in: J Clin Microbiol 1998 Sep;36(9):2801.

1997 Ausina V, Gamboa F, Gazapo E, Manterola JM, Lonca J, Matas L, Manzano JR, Rodrigo C, Cardona PJ, Padilla E. "Evaluation of the semiautomated Abbott LCx Mycobacterium tuberculosis assay for direct detection of Mycobacterium tuberculosis in respiratory specimens". J Clin Microbiol. 1997 Aug;35(8):1996-2002.

1997 Gamboa F, Manterola JM, Lonca J, Matas L, Viñado B, Giménez M, Cardona PJ, Padilla E, Ausina V. "Detection and identification of mycobacteria by amplification of RNA and DNA in pretreated blood and bone marrow aspirates by a simple lysis method". J Clin Microbiol. 1997 Aug;35(8):2124-8.

1997 Gamboa F, Manterola JM, Lonca J, Viñado B, Matas L, Giménez M, Manzano JR, Rodrigo C, Cardona PJ, Padilla E, Dominguez J, Ausina V. "Rapid detection of Mycobacterium tuberculosis in respiratory specimens, blood and other non-respiratory specimens by amplification of rRNA". Int J Tuberc Lung Dis. 1997 Dec;1(6):542-55.

1998 Gamboa F, Fernandez G, Padilla E, Manterola JM, Lonca J, Cardona PJ, Matas L, Ausina V. "Comparative evaluation of initial and new versions of the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test for direct detection of Mycobacterium

tuberculosis in respiratory and nonrespiratory specimens". J Clin Microbiol. 1998 Mar;36(3):684-9.

1998 Gamboa F, Dominguez J, Padilla E, Manterola JM, Gazapo E, Lonca J, Matas L, Hernandez A, Cardona PJ, Ausina V. "Rapid diagnosis of extrapulmonary tuberculosis by ligase chain reaction amplification". J Clin Microbiol. 1998 May;36(5):1324-9.

1998 Gamboa F, Manterola JM, Lonca J, Matas L, Cardona PJ, Padilla E, Viñado B, Domínguez J, Hernández A, Ausina V. "Comparative evaluation of two commercial assays for direct detection of Mycobacterium tuberculosis in respiratory specimens." Eur J Clin Microbiol Infect Dis. 1998 Mar;17(3):151-7.

1998 Gamboa F, Cardona PJ, Manterola JM, Lonca J, Matas L, Padilla E, Manzano JR, Ausina V. "Evaluation of a commercial probe assay for detection of rifampin resistance in Mycobacterium tuberculosis directly from respiratory and nonrespiratory clinical samples". Eur J Clin Microbiol Infect Dis. 1998 Mar;17(3):189-92.

1998 Matas L, Domínguez J, De Ory F, García N, Galí N, Cardona PJ, Hernández A, Rodrigo C, Ausina V. "Evaluation of Meridian ImmunoCard Mycoplasma test for the detection of Mycoplasma pneumoniae-specific IgM in paediatric patients". Scand J Infect Dis. 1998;30(3):289-93.

1998 Manterola JM, Gamboa F, Padilla E, Lonca J, Matas L, Hernández A, Giménez M, Cardona PJ, Viñado B, Ausina V. "Comparison of a nonradiometric system with Bactec 12B and culture on egg-based media for recovery of mycobacteria from clinical specimens". Eur J Clin Microbiol Infect Dis. 1998 Nov;17(11):773-7.

1999 Monsó E, Rosell A, Bonet G, Manterola J, Cardona PJ, Ruiz J, Morera J. "Risk factors for lower airway bacterial colonization in chronic bronchitis". Eur Respir J. 1999 Feb;13(2):338-42.

1999 Cardona PJ, Cooper A, Luquín M, Ariza A, Filipo F, Orme IM, Ausina V. "The intravenous model of murine tuberculosis is less pathogenic than the aerogenic model owing to a more rapid induction of systemic immunity". Scand J Immunol. 1999 Apr;49(4):362-6.

2000 Cardona PJ, Ausina V. "Delayed-type hypersensitivity and caseous necrosis in tuberculous granuloma. New ideas for the design of a new vaccine against human tuberculosis". Med Clin (Barc). 2000 Oct 21;115(13):503-9.

2000 Cardona PJ, Llatjós R, Gordillo S, Díaz J, Ojanguren I, Ariza A, Ausina V. "Evolution of granulomas in lungs of mice infected aerogenically with Mycobacterium tuberculosis". Scand J Immunol. 2000 Aug;52(2):156-63.

2000 Cardona PJ, Ausina V. "Histopathology of tuberculosis. Approximation to the clinical course of lung lesions in animal experimentation models induced with aerosols." Arch Bronconeumol. 2000 Dec;36(11):645-50.

2001 Cardona PJ, Llatjós R, Gordillo S, Díaz J, Viñado B, Ariza A, Ausina V. "Towards a 'human-like' model of tuberculosis: intranasal inoculation of LPS induces intragranulomatous lung necrosis in mice infected aerogenically with Mycobacterium tuberculosis". Scand J Immunol. 2001 Jan;53(1):65-71.

2002 Cardona PJ, Julián E, Vallès X, Gordillo S, Muñoz M, Luquin M, Ausina V. "Production of antibodies against glycolipids from the Mycobacterium tuberculosis cell wall in aerosol murine models of tuberculosis". Scand J Immunol. 2002 Jun;55(6):639-45.

2003 Cardona PJ, Gordillo S, Díaz J, Tapia G, Amat I, Pallarés A, Vilaplana C, Ariza A, Ausina V. "Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with Mycobacterium tuberculosis". Infect Immun. 2003 Oct;71(10):5845-54.

2003 Cardona PJ. "Advantages and limitations of experimental animal models for the study of infectious diseases". Enferm Infecc Microbiol Clin. 2003 Aug-Sep;21(7):327-8.

2003 Cardona PJ, Gordillo S, Amat I, Díaz J, Lonca J, Vilaplana C, Pallarés A, Llatjós R, Ariza A, Ausina V. "Catalase-peroxidase activity has no influence on virulence in a murine model of tuberculosis". Tuberculosis (Edinb). 2003;83(6):351-9.

2004 Cardona PJ, Ruiz-Manzano J. "On the nature of Mycobacterium tuberculosis-latent bacilli". Eur Respir J. 2004 Dec;24(6):1044-51.

2005 Cardona PJ, Amat I, Gordillo S, Arcos V, Guirado E, Díaz J, Vilaplana C, Tapia G, Ausina V. "Immunotherapy with fragmented Mycobacterium tuberculosis cells increases the effectiveness of chemotherapy against a chronical infection in a murine model of tuberculosis". Vaccine. 2005 Feb 3;23(11):1393-8.

2005 Garcia de Viedma D, Lorenzo G, Cardona PJ, Rodriguez NA, Gordillo S, Serrano MJ, Bouza E. "Association between the infectivity of Mycobacterium tuberculosis strains and their efficiency for extrarespiratory infection". J Infect Dis. 2005 Dec 15;192(12):2059-65.

2006 Cardona PJ, Soto CY, Martín C, Giquel B, Agustí G, Andreu N, Guirado E, Sirakova T, Kolattukudy P, Julián E, Luquin M. "Neutral-red reaction is related to virulence and cell wall methyl-branched lipids in Mycobacterium tuberculosis". Microbes Infect. 2006 Jan;8(1):183-90. Epub 2005 Aug 10. Erratum in: Microbes Infect. 2006 Sep;8(11):2668. Andreu, Núria [added].

2006 Gil O, Guirado E, Gordillo S, Díaz J, Tapia G, Vilaplana C, Ariza A, Ausina V, Cardona PJ. " Intragranulomatous necrosis in lungs of mice infected by aerosol with

Mycobacterium tuberculosis is related to bacterial load rather than to any one cytokine or T cell type". Microbes Infect. 2006 Mar;8(3):628-36.

2006 Cardona PJ. "RUTI: a new chance to shorten the treatment of latent tuberculosis infection".Tuberculosis (Edinb). 2006 May-Jul;86(3-4):273-89.

2006 Martin C, Williams A, Hernandez-Pando R, Cardona PJ, Gormley E, Bordat Y, Soto CY, Clark SO, Hatch GJ, Aguilar D, Ausina V, Gicquel B. "The live Mycobacterium tuberculosis phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs". Vaccine. 2006 Apr 24;24(17):3408-19.

2006 Tjärnlund A, Rodríguez A, Cardona PJ, Guirado E, Ivanyi J, Singh M, Troye-Blomberg M, Fernández C. "Polymeric IgR knockout mice are more susceptible to mycobacterial infections in the respiratory tract than wild-type mice". Int Immunol. 2006 May;18(5):807-16.

2006 Guirado E, Gordillo S, Gil O, Díaz J, Tapia G, Vilaplana C, Ausina V, Cardona PJ. "Intragranulomatous necrosis in pulmonary granulomas is not related to resistance against Mycobacterium tuberculosis infection in experimental murine models induced by aerosol". Int J Exp Pathol. 2006 Apr;87(2):139-49.

2006 Rodríguez-Güell E, Agustí G, Corominas M, Cardona PJ, Casals I, Parella T, Sempere MA, Luquin M, Julián E. "The production of a new extracellular putative long-chain saturated polyester by smooth variants of Mycobacterium vaccae interferes with Th1-cytokine production". Antonie Van Leeuwenhoek. 2006 Jul;90(1):93-108.

2006 Guirado E, Amat I, Gil O, Díaz J, Arcos V, Caceres N, Ausina V, Cardona PJ. "Passive serum therapy with polyclonal antibodies against Mycobacterium tuberculosis protects against post-chemotherapy relapse of tuberculosis infection in SCID mice". Microbes Infect. 2006 Apr;8(5):1252-9.

2006 Gordillo S, Guirado E, Gil O, Díaz J, Amat I, Molinos S, Vilaplana C, Ausina V, Cardona PJ. "Usefulness of acr expression for monitoring latent Mycobacterium tuberculosis bacilli in 'in vitro' and 'in vivo' experimental models". Scand J Immunol. 2006 Jul;64(1):30-9.

2006 Tjärnlund A, Guirado E, Julián E, Cardona PJ, Fernández C. "Determinant role for Toll-like receptor signalling in acute mycobacterial infection in the respiratory tract". Microbes Infect. 2006 Jun;8(7):1790-800.

2006 Cardona PJ, Amat I. "Origin and development of RUTI, a new therapeutic vaccine against Mycobacterium tuberculosis infection". Arch Bronconeumol. 2006 Jan;42(1):25-32.

2006 Cardona PJ. "Mycobacterium tuberculosis and Homo sapiens: a long road to cross". Med Clin (Barc). 2006 May 27;126(20):771-3.

2007 Cardona PJ. "Robert Koch was right. Towards a new interpretation of tuberculin therapy". Enferm Infecc Microbiol Clin. 2006 Jun-Jul;24(6):385-91. Spanish. Erratum in: Enferm Infecc Microbiol Clin. 2007 Feb;25(2):81.

2007 Cardona PJ. "New insights on the nature of latent tuberculosis infection and its treatment". Inflamm Allergy Drug Targets. 2007 Mar;6(1):27-39.

2007 Nobrega C, Cardona PJ, Roque S, Pinto do O P, Appelberg R, Correia-Neves M. "The thymus as a target for mycobacterial infections". Microbes Infect. 2007 Nov-Dec;9(14-15):1521-9.

2007 Cardona PJ. "Therapeutic vaccines against tuberculosis: a glowing future". Arch Bronconeumol. 2007 Nov;43(11):591-3.

2008 Rodríguez-Güell E, Agustí G, Corominas M, Cardona PJ, Luquin M, Julián E. "Mice with pulmonary tuberculosis treated with Mycobacterium vaccae develop strikingly enhanced recall gamma interferon responses to M. vaccae cell wall skeleton". Clin Vaccine Immunol. 2008 May;15(5):893-6.

2008 Vilaplana C, Ruiz-Manzano J, Gil O, Cuchillo F, Montané E, Singh M, Spallek R, Ausina V, Cardona PJ. "The tuberculin skin test increases the responses measured by T cell interferon-gamma release assays". Scand J Immunol. 2008 Jun;67(6):610-7.

2008 Guirado E, Gil O, Cáceres N, Singh M, Vilaplana C, Cardona PJ. "Induction of a specific strong polyantigenic cellular immune response after short-term chemotherapy controls bacillary reactivation in murine and guinea pig experimental models of tuberculosis". Clin Vaccine Immunol. 2008 Aug;15(8):1229-37.

2008 Di Liberto D, Locati M, Caccamo N, Vecchi A, Meraviglia S, Salerno A, Sireci G, Nebuloni M, Caceres N, Cardona PJ, Dieli F, Mantovani A. "Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection". J Exp Med. 2008 Sep 1;205(9):2075-84.

2008 Cardona PJ. "Should an immunosuppressed patient with a positive TIGRA test and a negative PPD test be treated?". Med Clin (Barc). 2008 May 31;130(20):776-7. Erratum in: Med Clin (Barc). 2008 Oct 25;131(14):525.

2008 Gil O, Vilaplana C, Guirado E, Díaz J, Cáceres N, Singh M, Cardona PJ. "Enhanced gamma interferon responses of mouse spleen cells following immunotherapy for tuberculosis relapse". Clin Vaccine Immunol. 2008 Nov;15(11):1742-4.

2008 Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, Daffé M, Emile JF, Marchou B, Cardona PJ, de Chastellier C, Altare F. "Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for M. tuberculosis persistence". PLoS Pathog. 2008 Nov;4(11):e1000204.

2009 Cáceres N, Tapia G, Ojanguren I, Altare F, Gil O, Pinto S, Vilaplana C, Cardona PJ. "Evolution of foamy macrophages in the pulmonary granulomas of experimental tuberculosis models". Tuberculosis (Edinb). 2009 March (89); 175-182.

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1997 Grant FEPAR of Basic research: "Estudio de la respuesta humoral frente antígenos glicolipídicos en un modelo de tuberculosis murina".

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Awards:

1999 Extraordinary award of the Genetics and Microbiology Department (Universitat Autònoma de Barcelona, Spain) to the thesis.

2000 VI Premi Bages de Ciències Mèdiques granted by the Acadèmia de Ciències Mèdiques de Catalunya i de Balears.

2002 Best paper award in the X Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica: "Desarrollo de un modelo humanizado de tuberculosis murina mediante la inoculación local de LPS mediante aerosol. Implicaciones de la dotación H-2 y la susceptibilidad de las cepas de ratones a la infección".

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OTHER ACHIEVEMENTS RELATED WITH THE PROFESSIONAL ACTIVITY

Spanish expert of the European Medicines Agency (EMEA) related to the regulation of antituberculous drugs. From 2006 up to date.

OTHER ACHIEVEMENTS RELATED WITH THE QUALITY OF EDUCATION

Acreditation to work as a General Medical Doctor in the Spanish National Health System, the public community health systems of the other countries member of the European Community.

6-month stage in the College of Veterinary Medicine and Biomedical Science of the Colorado State University to learn experimental tuberculosis models.

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Mycobacterial Antigens Exacerbate Disease Manifestations in *Mycobacterium tuberculosis*-Infected Mice

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To control tuberculosis worldwide, the burden of adult pulmonary disease must be reduced. Although widely used, *Mycobacterium bovis* BCG vaccination given at birth does not protect against adult pulmonary disease. Therefore, postexposure vaccination of adults with mycobacterial antigens is being considered. We examined the effect of various mycobacterial antigens on mice with prior *M. tuberculosis* infection. Subcutaneous administration of live or heat-treated BCG with or without lipid adjuvants to infected mice induced increased antigen-specific T-cell proliferation but did not reduce the bacterial load in the lungs and caused larger lung granulomas. Similarly, additional mycobacterial antigen delivered directly to the lungs by aerosol infection with viable *M. tuberculosis* mixed with heat-killed *Mycobacterium tuberculosis* (1:1) also did not reduce the bacillary load but caused increased expression of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which was associated with larger granulomas in the lungs. When *M. tuberculosis*-infected mice were treated with recombinant BCG that secreted cytokines shown to reduce disease in a preinfection vaccine model, the BCG secreting TNF-α, and to a lesser extent, IL-2 and gamma interferon (IFN-γ), caused a significant increase in granuloma size in the lungs. Moreover, treatment of *M. tuberculosis*-infected mice with recombinant murine TNF-α resulted in increased inflammation in the lungs and accelerated mortality without affecting the bacillary load. Taken together, these studies suggest that administration of mycobacterial antigens to mice with prior *M. tuberculosis* infection leads to immune activation that may exacerbate lung pathology via TNF-α-induced inflammation without reducing the bacillary load.

The idea of immune modulation to treat tuberculosis is not new. In 1890, at the World Congress of Medicine, Robert Koch announced that he had prepared "substances" that completely cured guinea pigs in the late stages of tuberculosis (TB) (14). The substance, later called "old tuberculin," was a glycerin extracted filtrate of cultures of the tubercle bacillus. With much fanfare, patients were treated with this bacterial extract. Unfortunately, the treatment caused florid local and systemic reactions in many of the patients with relatively mild disease. In addition, of the 230 patients with advanced cavitary disease who received this treatment, 30 died. This intense reaction to extracts of *Mycobacterium tuberculosis* and the associated clinical worsening became known as the "Koch phenomenon" (11).

The practical implications of the Koch phenomenon should be reconsidered in light of recent efforts to develop a new anti-TB vaccine. The goal of vaccination is protection against adult pulmonary TB. Neonatal *Mycobacterium bovis* BCG vaccination, which is widely used throughout the world, does not protect against adult pulmonary disease, even when it protects against the more severe forms of childhood TB (8). New strategies have therefore been proposed, including vaccination of adults with BCG or other mycobacterial preparations (7). The latter approach may be problematic. In areas of high endemicity, many individuals will have already been infected and may even have subclinical disease. If they now receive a strong immunogen, the ensuing host response may result in exacerbation of the occult disease leading to severe toxicities (Koch phenomenon).

The effect of administration of mycobacterial preparations on the outcome of disease has recently been studied by a number of investigators (1, 10, 21). Lowrie et al. showed that postexposure vaccination with either BCG or DNA encoding the *M. tuberculosis* heat shock protein Hsp70 or ESAT6 had little or no effect on bacillary load in the spleens or lungs of infected mice (16). However, repeated vaccination with DNA encoding *Mycobacterium leprae* Hsp65 reduced the number of bacilli in lungs and spleen by 1 $\log_{10}$ and 2 $\log_{10}$ at 2 and 5 months postintervention, respectively. In contrast, Turner and colleagues explored the use of two other new candidate vaccines (a subunit vaccine and a DNA vaccine, both containing *M. tuberculosis* Ag85) as immunotherapeutic agents in mice (23). When administered to mice already infected with *M. tuberculosis*, neither BCG nor these two candidate vaccines caused any improvement in the course of infection. Furthermore, repeated BCG vaccination of infected mice resulted in exacerbation of the granulomatous response in the lungs (23).

To further examine the effect of mycobacterium-induced immune activation on the host response during *M. tuberculosis*

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infection, we infected mice by aerosol with virulent *M. tuberculosis*. Then, 5 weeks later, the infected mice were inoculated with various preparations containing BCG or with BCG secreting murine cytokines. In addition, to determine whether the presence of additional antigen in the lungs might have an impact on outcome, naïve mice were infected by aerosol with live *M. tuberculosis* or a mixture of live and dead *M. tuberculosis*. Finally, *M. tuberculosis*-infected mice were treated directly with recombinant cytokines via intranasal delivery. The course of the infection was monitored by enumeration of bacilli in infected tissues, histologic examination of the lungs, quantitation of cytokine mRNA induced in the lungs, and peripheral T-cell proliferation in response to mycobacterial antigens.

MATERIALS AND METHODS

Mice. Seven- to 8-week-old female (B6 × D2)F_1 mice, free of common pathogens, were obtained from Charles River Laboratories, Wilmington, Mass., and housed in the BSL3 (Biosafety Level 3) Animal Facility of The Rockefeller University for the duration of these experiments. All protocols were approved by the Animal Use and Care Committee of The Rockefeller University.

Infection of mice. Aerosol infection was carried out according to a protocol developed in this laboratory (17). Briefly, mice were inoculated via the respiratory route by exposure to an aerosolized suspension of *M. tuberculosis* (see below) generated by a Lovelace nebulizer using a nose-only exposure system (In-Tox Products, Albuquerque, N.M.) (22). For each experiment 24 mice were exposed for 30 min to the aerosol. This procedure resulted in implantation of approximately 100 organisms into the lungs of mice, as confirmed by plating lung homogenates 3 h after infection. In some experiments *M. tuberculosis* was autoclaved and then mixed 1:1 with live bacilli prior to aerosol infection.

M. tuberculosis and *M. bovis* strains. *M. tuberculosis* strain Erdman was provided as multiple stock vials by J. Belisle, Colorado State University (Fort Collins, Colo.); *M. tuberculosis* H37Rv was from the Trudeau Mycobacterial Culture Collection (Trudeau Institute, Saranac Lake, N.Y.). The *M. tuberculosis* clinical isolate HN878 was provided by J. M. Musser (20). BCG was obtained from Statens Serum Institute, Copenhagen, Denmark (BCG vaccine SSI, batch 9854). BCG strain Montreal, which was used to generate recombinant BCG expressing murine cytokines, including BCG expressing gamma interferon (BCG–IFN-γ), tumor necrosis factor alpha (BCG–TNF-α), and interleukin 2 (BCG–IL-2) and the control strain of BCG, carrying the plasmid vector only (BCG vector), was obtained from Richard Young, The Whitehead Institute, Cambridge, Mass. (18). All mycobacteria were grown on Middlebrook 7H9 medium (Difco, Detroit, Mich.), and bacillary stocks were stored at 10^7 to 10^8 bacilli/ml and kept at −70°C until use.

BCG preparations for postinfection inoculation. Lyophilized BCG (SSI) (1.5 mg) was reconstituted in 2 ml of diluent (Sauton; Statens Serum Institute), according to the manufacturer's instructions (viable BCG). In addition, BCG was reconstituted in 200 μl of diluent as described above and was heated in a water bath at 60°C for 10 min. No growth of heat-treated BCG was noted using culture on egg-based solid media (Lowenstein-Jensen) or 4 weeks of growth in the BACTEC system (Bactec 460; Becton Dickinson, Sparks, Md.). L3 adjuvant was prepared using 0.62 mg of solid monooleate (analytic grade; Kebo AB, Stockholm, Sweden), 0.48 mg of oleic acid (analytic grade; Kebo AB), and 180 mg of soybean oil (pharmaceutical grade; Karlshamn AB, Karlshamn, Sweden) and was liquefied by gentle heat at 30°C (manuscript in preparation). For injection, 200 μl of the heat-treated BCG was mixed in the L3 adjuvant, probe sonicated for 10 to 15 s, and brought to volume (2.25 ml) with 0.1 M Tris buffer, pH 7.5 (heat-treated BCG). For boosting of mice, 200 μl of heat-treated BCG was mixed in half the amount of L3 adjuvant (see above) and brought to volume (2.25 ml) with 0.1 M Tris buffer, pH 7.5.

Vaccination of infected mice with BCG preparations. Five weeks after the initial infection with *M. tuberculosis* H37Rv, mice were divided into four groups. Groups 1 and 2 received 0.1 ml of viable BCG subcutaneously (s.c.). Group 3 received 0.1 ml of heat-treated BCG in L3 adjuvant s.c., while group 4 received no treatment. Three weeks later, groups 2 and 3 were boosted with heat-treated BCG in L3 adjuvant by intranasal administration of 10 μl (5 μl per nostril). Mice were evaluated at 12 weeks. In another experiment, either 6 weeks before or 5 weeks after the aerosol infection, mice were vaccinated s.c. with one of the recombinant BCG strains (BCG–IFN-γ, BCG–TNF-α, BCG–IL-2, or BCG vector) (18) at a dose of 10^6 organisms per mouse. Three weeks after the initial vaccination, the mice received a boost with the same dose of recombinant BCG. A control group of mice received no BCG. Mice were evaluated at 12 and 20 weeks postchallenge with *M. tuberculosis*.

Intranasal treatment of infected mice. Recombinant murine TNF-α was obtained from Endogen (Boston, Mass.), reconstituted in 1% bovine serum albumin–phosphate-buffered saline to a final concentration of 2×10^5 IU/ml, and kept at 4°C. The cytokine was administered intranasally for 5 consecutive days per week for 4 weeks (6). Five microliters was applied directly to the nostrils of each mouse (final dose of TNF-α, 10^3 IU per mouse per day). Recombinant murine IFN-γ was obtained from Valbiotech (Paris, France) and reconstituted in 1% bovine serum albumin–phosphate-buffered saline to a final concentration of 10^6 IU/ml. Ten microliters was applied into the nostrils of each mouse as described above (final dose of IFN-γ, 10^4 IU per mouse per day). Control mice were untreated.

CFU assay. The number of viable mycobacteria in lungs, livers, and spleens of infected mice were evaluated at designated time points. Tenfold serial dilutions of organ homogenates were plated onto 7H11 agar (Becton Dickinson) and were incubated at 37°C. The number of viable bacilli was evaluated by counting individual colonies after 2 to 3 weeks of growth.

Cytokine mRNA levels in the infected lung. Total cellular RNA from lungs of infected mice was obtained at designated time points following aerosol infection. Tissues were homogenized in 3 ml of RNAzolB (Cinna/Biotex Lab. Inc., Houston, Tex.), and RNA was extracted according to the manufacturer's instructions. The reverse transcription-PCR was carried out as previously described (15). Briefly, 1 μg of RNA was reverse transcribed using a Moloney murine leukemia virus reverse transcriptase and was amplified with *Taq* polymerase according to procedures given in the GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, N.J.). Primers for cytokines and β-actin were used as described (17). Densitometry of the amplified bands was carried out using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Results were normalized to the density of β-actin.

Lymphocyte proliferation assay. Spleen and draining lymph nodes were removed at each time point and were processed as described (4). Briefly, cells isolated from spleen and lymph nodes were cultured at 2×10^5 cells/100 μl in 96-well U-bottom plates in RPMI 1640 medium (Gibco BRL, Gaithersburg, Md.) and were supplemented with 10% fetal bovine serum, penicillin, and streptomycin (final concentration, 50 μg/ml) (all obtained from Gibco BRL). The cells were incubated with concanavalin A (final concentration, 5 μg/ml) (Sigma, St. Louis, Mo.) or *M. tuberculosis* H37Ra sonicate (final concentration, 50 μg/ml) at 37°C for 3 or 5 days, respectively, and were then pulsed with [^{3}H]thymidine (1 μCi/well) for an additional 18 h. Incorporation of [^{3}H]thymidine was measured by β-scintillation counting. Values were expressed as mean counts per minute in the cultures.

Histopathology. At various time points after infection, lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for histology. Sections were stained with hematoxylin and eosin and Ziehl-Neelsen for histologic evaluation and photography.

Morphometric evaluation of granuloma size. Morphometry of the lesions was performed using Microcomp, a computer-based image analysis system (Southern Micro Institute, Atlanta, Ga.) and/or Sigmascan Pro 5.0 (SPSS Science Inc., Chicago, Ill.). A calibration micrometer (in square micrometers) slide was used to determine the area evaluated.

Statistical analysis. Data were analyzed using an independent Student *t* test. A P of <0.05 was considered statistically significant. Kaplan-Meier analysis was used to determine statistical significance of the differences in survival time of mice.

RESULTS

The effect of postinfection exposure to mycobacterial antigens. To determine whether the course of established disease is altered by immune stimulation with mycobacterial antigens, mice were first infected by aerosol with 100 CFU of *M. tuberculosis* H37Rv. At 5 weeks after infection, prior to any additional treatment, the number of CFU in the lungs was approximately 6.6 $\log_{10}$. At this time, infected mice were split into four groups, and mice in groups 1 and 2 were vaccinated with viable BCG while mice in group 3 received heat-treated BCG in L3 adjuvant. Three weeks later mice in groups 2 and 3 were boosted with heat-treated BCG in L3 adjuvant, as described

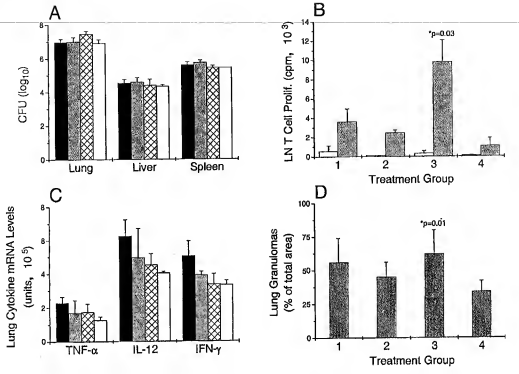

FIG. 1. Effect of postinfection vaccination on bacillary load (CFU) (A), lymph node (LN) T-cell proliferation (B), lung cytokine mRNA levels (C), and area of lung occupied by granulomas (D). (A and C) *M. tuberculosis*-infected mice were vaccinated according to the protocol described in Materials and Methods. Group 1, viable BCG (black bar); group 2, viable BCG followed by boost with heat-treated BCG in L3 adjuvant (gray bar); group 3, heat-treated BCG in L3 adjuvant and boosted as above (hatched bar); group 4, no vaccination (empty bar). (B) Lymph node cells stimulated with H37Ra sonicate (gray bars) or unstimulated control (empty bar). (D) Percentage of lung parenchyma occupied by granuloma. Results are from four animals per group at 12 weeks post-initial infection, expressed as means ± standard deviation. *, P compared to unvaccinated controls (group 4).

above (Materials and Methods). Four weeks later (at 12 weeks after the initial infection), the numbers of CFU in lungs, livers, and spleens of mice in all four groups were evaluated and found to be similar (Fig. 1A). Thus, postinfection immunotherapy with these BCG preparations had no effect on the bacillary loads in the organs of any of the infected mice.

An evaluation of the systemic T-cell response of the infected mice was also carried out. Cells were isolated from the draining lymph nodes of infected and vaccinated mice at 12 weeks and stimulated in vitro with *M. tuberculosis* H37Ra sonicate. Compared to the control infected mice (group 4), an increased proliferative response was noted in all treatment groups and was significant for group 3 (Fig. 1B). Lymph node cells from infected animals that received heat-treated BCG in L3 adjuvant followed by intranasal boosting with heat-treated BCG in L3 adjuvant (group 3) showed the largest increase in proliferative responses in vitro compared to the other treatment group and the unvaccinated controls ($P = 0.03$). Cells isolated from the spleens of the infected, vaccinated animals showed similar patterns of proliferative responses, although these were much lower (not shown). To examine the immune stimulation in the lungs of infected, vaccinated animals, cytokine mRNA was prepared from the lungs at 12 weeks. When compared to the infected, unvaccinated controls (group 4), the vaccinated, infected animals (groups 1 to 3) had only slightly higher levels of TNF-α mRNA in the lungs (Fig. 1C). Animals in group 1 also had somewhat higher levels of mRNAs for IL-12 and IFN-γ; all these differences were not statistically significant.

When the lungs of the mice in the four treatment groups were examined histologically at week 12, granulomas were larger in the infected animals that had received heat-treated BCG in L3 adjuvant and were then boosted intranasally (group 3). Morphometric analysis revealed that 69% of the lung parenchyma was occupied by granulomas, compared to 56% in group 1, 47% in group 2, and 36% in the control animals (group 4) (Fig. 1D). Thus, although the levels of systemic immune activation as measured by antigen-specific T-cell proliferation increased, the vaccine-induced immune stimulation did not affect the number of CFU in the lungs. However, the immune activation was associated with an exacerbation of pathology, as indicated by the size of the granulomas in the lungs (compare Fig. 1B and D).

Effect of additional antigen on the host response to *M. tuberculosis* infection. To examine whether the presence of additional mycobacterial antigen in the infected lung changed the course of infection, mice were infected with either viable *M. tuberculosis* strain Erdman (100 CFU) or the same dose of viable bacilli (100 CFU) mixed with an equivalent dose of heat-killed organisms (1:1). The presence of additional heat-killed organisms in the infecting inoculum had no significant effect on the subsequent bacillary load (CFU) in the lung, spleen, and liver of infected mice (Table 1). In mice that

TABLE 1. Infection with viable and viable plus heat-killed *M. tuberculosis*

Type of infection and no. of wk	No. of CFU[a] (log_{10}) in:			Amt of lung cytokine mRNA[a] (units) for:	
	Lung	Spleen	Liver	TNF-α	IL-6
Viable bacilli					
1	3.7 ± 0.1	0	1.4 ± 1.2	63 ± 22	46 ± 10
2	5.2 ± 0.3	1.6 ± 1.5	1.9 ± 1.6	69 ± 30	174 ± 17
3	5.5 ± 0.1	3.9 ± 0.2	3.2 ± 0.6	144 ± 53	233 ± 33
4	6.0 ± 0.3	4.1 ± 0.1	2.6 ± 0.3	151 ± 51	161 ± 15
Viable + heat-killed bacilli					
1	3.5 ± 0.4	0	1.8 ± 1.7	33 ± 5	46 ± 5
2	5.1 ± 0.2	0.9 ± 0.5	2.8 ± 0.8	111 ± 9	243 ± 25
3	5.5 ± 0.1	3.6 ± 0.2	3.2 ± 0.2	269 ± 24[b]	384 ± 37[b]
4	5.8 ± 0.2	3.9 ± 0.6	3.4 ± 0.2	132 ± 81	149 ± 27

[a] Number of mice per group per time point ($n = 5$).
[b] $P < 0.05$ compared to infection with viable bacilli only.

received the live infection only, levels of mRNA for TNF-α and for IL-6 increased over the first 3 weeks of infection (Table 1). When mice were infected with the mixture of live plus dead *M. tuberculosis*, even higher expression of cytokine mRNA for TNF-α and for IL-6 was noted. The difference was most pronounced at week 3 ($P < 0.05$); by week 4 the difference was no longer seen (Table 1). In the lungs of mice infected with the mixture of viable and heat-killed bacilli, the higher cytokine mRNA levels were associated with larger granulomas (Fig. 2). In the mice infected with live plus dead organisms, the mean size of lesions at 4 weeks postinfection was 6.17 mm^3 ± 1.3 mm^3, compared to 4.38 mm^3 ± 0.64 mm^3 in those infected with live organisms only. Distended alveolar spaces (edematic lung) were also seen, suggesting a relative loss of lung function (Fig. 2). These results indicate that the additional mycobacterial antigen in the lungs of mice receiving the mixed infection induces a stronger local inflammatory response (increased TNF-α and IL-6 production; Table 1), leading to increased lung pathology without altering the bacillary burden.

Effect of vaccination with recombinant BCG secreting murine cytokines on the bacillary load and granuloma size in infected organs. To determine whether particular cytokines could enhance the BCG-induced protective immune response to *M. tuberculosis*, mice were vaccinated s.c. with recombinant BCG secreting murine cytokines (BCG vector, BCG–TNF-α, BCG–IFN-γ, or BCG–IL-2) followed by *M. tuberculosis* H37Rv challenge, as described (Materials and Methods). In these experiments, the recombinant BCG used was prepared from BCG strain Montreal, a known weak immunogen for mice (19). By 12 weeks after *M. tuberculosis* challenge, the bacilli in the lungs had grown to a concentration of 3×10^7 CFU in control (nonvaccinated) mice (Fig. 3, top). Vaccination with BCG vector resulted in a bacillary load reduced by about fourfold. Cytokine secretion by the recombinant BCG

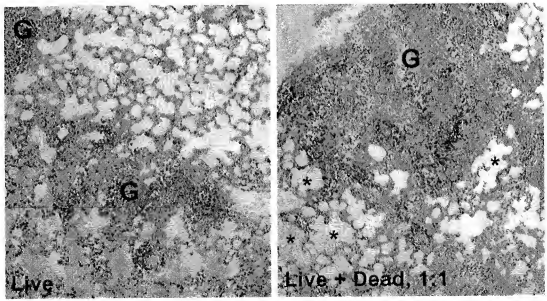

FIG. 2. Effect of additional antigen on the granulomatous response in the lungs of mice at 3 weeks post-aerosol infection with viable *M. tuberculosis* (live) or viable plus heat-killed *M. tuberculosis* (live + dead, 1:1). G, granuloma; *, distended alveolar spaces (edematic lung). Ziehl-Neelsen stain, magnification, ×10.

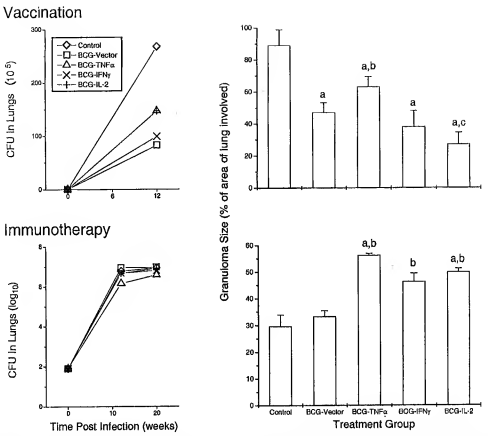

FIG. 3. Effect of vaccination (preinfection, top) and immunotherapy (postinfection, bottom) with cytokine-secreting BCG on number of CFU in lung at 12 and 20 weeks post-initial infection (left panels) and on granuloma size at 12 weeks (right panels). Results are from four animals per group per time point, expressed as means ± standard deviation. a, $P < 0.05$ compared to unvaccinated controls; b, $P < 0.05$, higher than for BCG vector-vaccinated mice; c, $P < 0.05$, lower than for BCG vector-vaccinated mice.

did not improve the control of the bacillary growth in the lungs. At 12 weeks postinfection, the lungs of unvaccinated controls showed an extensive inflammatory infiltrate composed of macrophages, lymphocytes, and neutrophils within the alveolar space consistent with a pneumonic process in addition to large, coalescent granulomas. The size of the granulomas was reduced by about 50% in BCG vector-vaccinated mice compared to unvaccinated controls ($P < 0.05$) (Fig. 3, top). Vaccination with BCG secreting IL-2 or BCG secreting IFN-γ resulted in even smaller granulomas than the BCG vector-vaccinated mice ($P < 0.05$). However, BCG–TNF-α vaccination resulted in larger granulomas than did vaccination with BCG vector ($P < 0.05$), though the granulomas were still smaller than in nonvaccinated mice ($P < 0.05$). Thus, of the cytokines tested, IL-2 together with BCG appeared to impart the most protection in terms of limiting immunopathology in the lungs; the presence of TNF-α appeared to exacerbate the pathology.

Effect of postinfection vaccination with cytokine-producing BCG. To determine whether particular cytokines could render BCG vaccination more effective against ongoing *M. tuberculosis* infection, recombinant strains of BCG producing various murine cytokines were used to treat infected mice. Mice infected with H37Rv were vaccinated twice (at 5 and 8 weeks) postinfection with 10^6 organisms of viable BCG–TNF-α, BCG–IL-2, BCG–IFN-γ, or BCG vector. No effect on the number of CFU in the lungs or spleens was observed at weeks 12 and 20 postinfection compared to in the organs of unvaccinated, infected mice (Fig. 3, bottom). Postinfection administration of recombinant BCG secreting cytokines did, however, affect the size of the granulomas. Larger granulomas involving 56.2% of the lung were observed in the infected mice that had received recombinant BCG–TNF-α, compared to 29.6% in the control unvaccinated mice ($P = 0.02$) (Fig. 3, bottom). Infected mice treated with BCG–IL-2 or BCG–IFN-γ had intermediate-sized granulomas (50 or 46.4%, respectively). Thus, the combination of the immune-stimulatory cytokines with BCG resulted in exacerbation of lung pathology; the worst effect was seen when BCG was combined with TNF-α.

Effect of exogenous TNF-α on course of *M. tuberculosis* infection. The previous experiments suggested that the deleterious component of the immune response to mycobacterial antigens is excess TNF-α. To directly examine the effect of excess TNF-α on lung pathology, mice infected with 80 CFU of *M. tuberculosis* were treated intranasally with recombinant murine TNF-α for the first 4 weeks of infection. At 4 weeks and then 8 weeks after infection and treatment, the numbers of CFU in lungs and spleens were similar (Fig. 4, top). However, survival of the mice was substantially altered. Mice infected with *M.*

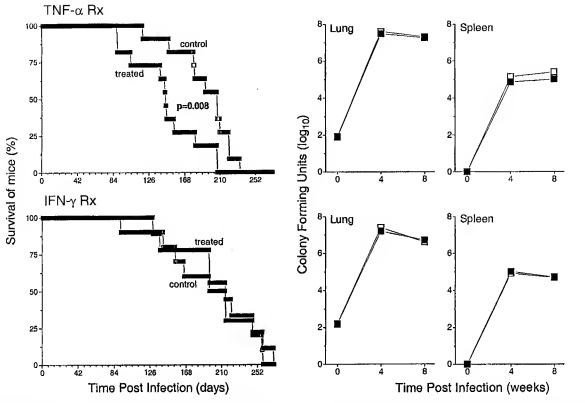

FIG. 4. Effect of exogenous recombinant cytokines on the bacillary load (right panels) and survival of infected mice (left panels). Mice were infected with *M. tuberculosis* and treated intranasally with recombinant murine TNF-α (top panels) or IFN-γ (bottom panels). Solid squares, cytokine-treated mice; empty squares, control untreated mice. Results for survival are from 11 mice per group and for numbers of CFU from four mice per group per time point.

tuberculosis and treated with TNF-α succumbed to the infection significantly earlier than mice that had not received the cytokine treatment ($P = 0.008$) (Fig. 4, top). In contrast, when infected mice (100 CFU at baseline) were treated intranasally with IFN-γ, no effect on survival or number of CFU in lungs and spleens was noted (Fig. 4, bottom).

TNF-α treatment of infected mice was associated with increased cellularity of the lungs, including higher numbers of polymorphonuclear leukocytes in the granulomas and larger numbers of foamy macrophages in the alveolar spaces at 4 weeks (Fig. 5). The macrophages did not appear to be more activated, as there was no increase in the mRNA levels of inflammatory cytokines in the lungs (IL-6, IL-10, IL-12, and TNF-α) (not shown). The lungs of the infected, untreated mice appeared to have higher numbers of small lymphocytes (Fig. 5). Again, the lymphocytes did not appear to be more activated, since the IFN-γ mRNA levels were similar or even somewhat lower (about 40%) in the lungs of TNF-α-treated mice at 4 and 8 weeks (not shown). Thus, TNF-α treatment appeared to result in the exacerbation of lung pathology without significant changes in the bacillus burden. On the other hand, IFN-γ treatment did not affect these parameters.

DISCUSSION

Our results, as well as those of others, demonstrate how difficult it is to change the course of existing *M. tuberculosis* infection in the lungs by immunotherapy with vaccines. Indeed, Koch himself did not claim that his treatment with "tuberculin lymph" killed the bacteria (5). In our postinfection experiments, vaccination with BCG, BCG plus other antigens, recombinant BCG secreting murine cytokines, or heat-killed *M. tuberculosis* did not reduce the bacterial load in the lungs of infected mice. Even those vaccines that were shown to be effective as preexposure vaccines (Fig. 3) failed to reduce the number of CFU when administerred as postinfection vaccines. Analogous results have been obtained by other investigators. Turner et al. tested three different vaccines in infected mice and guinea pigs as immunotherapy: (i) BCG Pasteur, (ii) a subunit vaccine containing purified culture filtrate proteins of *M. tuberculosis* emulsified in an adjuvant together with recombinant murine IL-2, and (iii) a DNA vaccine consisting of a vector expressing the gene for *M. tuberculosis* Ag85A (23). The candidate vaccines were selected because they had shown efficacy as preexposure vaccines in protecting naïve animals against challenge with *M. tuberculosis* (1, 10). However, all three of these vaccines, although protective as preexposure vaccines, failed to reduce the bacterial load in the lungs of mice with prior tuberculosis infection. Similarly, Lowrie et al. tested a number of DNA vaccines encoding *M. leprae* Hsp65 or *M. tuberculosis* Hsp70 or ESAT-6 as postinfection treatments (16). Only the DNA vaccine encoding *M. leprae* Hsp65 resulted in some reduction in bacterial load in the lungs of infected mice.

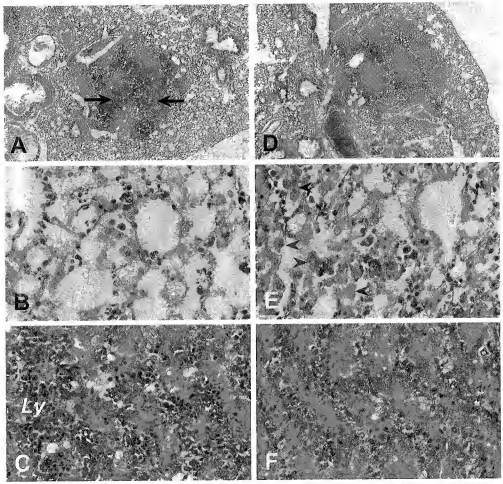

FIG. 5. Effect of exogenous TNF-α on the granulomatous response in the lungs. Mice were infected with *M. tuberculosis* and treated intranasally with recombinant murine TNF-α, and the lungs were examined at 4 weeks postinfection. (A to C) Untreated mice. (D to F) TNF-α-treated mice. Note extensive lymphocyte infiltration in arrows (A) and *Ly* (C). In response to TNF-α treatment (E), large macrophages are present in the alveolar spaces (arrowheads). Hematoxylin and eosin stain. (A and D) Magnification, ×4. (B, C, E, and F) Magnification, ×40.

Interestingly, when preexisting infection had been eliminated with antibiotic therapy, repeated postexposure vaccination did protect against reactivation of disease (16). Thus, it is possible that immune modulation in the presence of antituberculous therapy may facilitate bacillary clearance in the lungs (9). Indeed, in a previous pilot study of patients with multidrug-resistant pulmonary TB, we observed that administration of recombinant IL-2 as adjunctive therapy combined with anti-TB chemotherapy resulted in accelerated sputum clearance and improved radiographic appearance of the lungs (12).

As we have clearly shown here, increased immune activation is associated with exacerbation of pathology at the sites of preexisting TB. This had originally been noted by Koch. In response to the treatment with "tuberculin lymph," infected tissues became inflamed and necrotic and partially sloughed often in association with clinical worsening. The English physician and writer A. Conan Doyle wrote about Koch's treatment, "It may also be remarked that the fever after the injection is in some cases so very high (41°C) that it is hardly safe to use in the case of a debilitated patient" (in Review of Reviews, December 1890) (5). Our experiments in mice suggest that the cause of the worsening pathology and fever observed by Koch in patients may be excess TNF-α at the site of infection. In our murine experiments, the increased size and cellularity of the granulomas were accompanied by increased expression of TNF-α mRNA in the lung. Consistent with this, vaccination of mice with BCG–TNF-α resulted in an increase in the size of lung granulomas. Also, treatment of infected mice with recombinant murine TNF-α resulted in increased inflammation in the lung and accelerated death. In a previous study, in which mice were infected with BCG–TNF-α, we noted that high levels of TNF-α at the BCG infection site resulted in much severer lung pathology and decreased survival (2). Furthermore, TNF-α-associated clinical worsening has been observed in patients with severe TB upon initiation of anti-TB chemotherapy (3). This was probably due to death of the organisms and release of mycobacterial components. Mycobacteria and their products have previously been shown to induce TNF-α production through signaling of the macrophage Toll-like receptors (24, 25).

In addition to the safety issues associated with postinfection vaccination and the Koch phenomenon, there is also an oper-

ational consideration for any trial designed to test postexposure vaccine. Vaccination of adults in an area of high endemicity might reveal occult disease, which might confound interpretation of the results, since the number of TB cases in the vaccine arm would be selectively increased compared to that in the untreated arm. This may have been the case in the BCG vaccine trial carried out in the Karonga region of Malawi, where significantly higher rates of pulmonary TB were reported among scar-positive persons who had received a second dose of BCG (13). Thus, for both safety (immune-mediated pathology) and study design (subclinical disease scored as a newly acquired TB), any study of candidate postexposure vaccines must ensure that recruited participants do not have undiagnosed disease.

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L.T. and A.L.M. contributed equally to the paper.

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Effective Preexposure Tuberculosis Vaccines Fail To Protect When They Are Given in an Immunotherapeutic Mode

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Two vaccine formulations previously shown to induce protective immunity in mice and prevention of long-term necrosis in guinea pigs were tested as potential immunotherapeutic vaccines in mice earlier infected by aerosol with *Mycobacterium tuberculosis*. Neither vaccine had any effect on the course of the infection in the lungs, but both reduced the bacterial load in the spleen. Similarly, inoculation with *Mycobacterium bovis* BCG had no effect whatsoever and, if given more than once, appeared to induce an increasingly severe pyogranulomatous response in the lungs of these mice.

The current vaccine against tuberculosis, *Mycobacterium bovis* BCG, is cheap to produce and safe to administer, but it is far from effective, especially when the individual reaches the teenage years and above (15, 16). This lack of protective efficacy, now observed in several controlled clinical trials, has resulted in considerable effort being directed toward the construction of a new generation of tuberculosis vaccines (9–11).

Not only will this process be difficult, despite the innovative approaches that have already emerged, but the question remains as to when a new vaccine should be given and how its efficacy should then be monitored. The most obvious approach would be to vaccinate early in life, prior to any exposure to or infection with *Mycobacterium tuberculosis*, and then to monitor individuals for many decades to see if they then contract disease. Here the unanswered question is whether the vaccine should replace BCG, be given in tandem, or used to boost the immunity induced by the BCG vaccine.

There is an alternative approach recently suggested in which the vaccine is given as a postexposure or immunotherapeutic vaccine after the individual has been infected with *M. tuberculosis* (4). The idea here is that this type of vaccine not only will amplify the ongoing response but also may have other benefits such as overcoming aberrant responses to other "environmental" mycobacteria which may compromise the capacity of the individual to respond adequately to pulmonary infection.

In the current study, we examined the possibility that two newly developed subunit and DNA vaccines, recently shown to be effective in the mouse and guinea pig models against pulmonary tuberculosis (1, 5), might have some effect if given as an immunotherapeutic vaccine. The results of the study show, however, that neither these materials, nor BCG itself, modulated the course of an aerosol infection with *M. tuberculosis*. Interestingly, however, both nonliving vaccines reduced bacterial counts in the spleen, either directly or by reducing dissemination from the lungs. Finally, in mice given additional inoculations with BCG, an increasingly severe pyogranulomatous response was seen in the lungs as the tuberculosis infection progressed.

Specific-pathogen-free female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, Mass.). Mice 6 to 8 weeks in age were kept in ABL-3 biohazard facilities throughout the study and maintained with sterile water, bedding, and mouse chow. *M. tuberculosis* Erdman was originally obtained from the Trudeau Mycobacteria Collection, Saranac Lake, N.Y. Bacteria were grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and frozen in aliquots at −70°C until needed. Animals were infected by the aerosol route with a low dose of bacteria; briefly, the nebulizer compartment of an airborne infection apparatus (Glas-Col, Terre Haute, Ind.) was filled with 5 ml of a suspension containing 10^7 bacteria to allow uptake of 50 to 100 viable bacteria per lung during a 30-min exposure. The numbers of viable bacteria in the lung, liver, and spleen were determined at various time points by plating serial dilutions of whole-organ homogenates onto nutrient Middlebrook 7H11 agar (Life Technologies, Gaithersburg, Md.) and counting bacterial colonies after 20 to 30 days of incubation at 37°C in humidified air. The data are expressed as $\log_{10}$ values of the mean numbers of bacteria recovered per organ ($n = 4$ animals).

Vaccination was administered twice, 50 and 73 days after infection; control mice were given 200 μl of saline subcutaneously. BCG (strain Pasteur) was given in a dose of 10^6 subcutaneously in 200 μl of sterile saline. Mid-log-phase culture filtrate proteins (CFP) from *M. tuberculosis* were prepared and purified as described previously (14) and then emulsified in MPL-TeoA adjuvant (Ribi Immunochem, Hamilton, Mont.) supplemented with 100 μg of recombinant interleukin 2 (IL-2) (Chiron Corp., Emeryville, Calif.). DNA vaccines consisting of the control plasmid vector V1Jns or V1Jns containing the gene encoding the secreted form of *M. tuberculosis* antigen 85A (Ag85A) protein (Ag85-DNA) were kindly provided by Merck Research Laboratories (West Point, Pa.). Mice were given 50 μg of plasmid DNA in saline by injection into a hind quadriceps muscle.

Lungs were harvested, fixed in formal saline, and then sectioned and stained with hematoxylin and eosin. Slides were read by a veterinary pathologist who lacked prior knowledge of the treatment groups.

As shown in Fig. 1, the infection grew progressively in the

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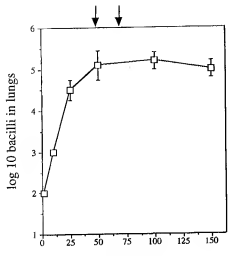

FIG. 1. Course of infection in the lungs of control mice. Data are shown as mean values ($n = 4$) ± standard errors of the means. Arrows indicate administration of immunotherapeutic vaccines to test groups.

lungs for approximately 30 days before entering into a chronic stage of disease in which the bacterial load did not fluctuate appreciably. The various vaccines and appropriate control inoculations were given on days 50 and 73, and potential changes in bacterial load were assayed on days 95 and 150. No differences were observed in lung loads at either time point, in any of the groups (Table 1). Interestingly, despite no apparent effects in the lungs, mice receiving the Ag85-DNA vaccine or the CFP-based vaccine both showed evidence of statistically significant reductions in splenic bacterial loads which were not seen in vaccine vehicle controls.

No histologic changes were seen in the lungs of mice given the experimental vaccines, with all animals exhibiting a typical multifocal lymphocytic granulomatous response after exposure to the challenge infection. In mice receiving BCG given after the tuberculosis infection, some adverse pathology was noted, and so in a second experiment, the BCG vaccine was given up to three times at 15-day intervals after challenge. In this experiment, the control mice again exhibited expected pathology as reported above (Fig. 2), but animals given BCG after the tuberculosis infection developed increasingly severe pyogranulomatous responses (lesions containing large numbers of neutrophils) with the degree of severity seeming to mirror the number of BCG inoculations. Such abscesses are known to have the potential to be fatal (3).

The results of this study show that immunotherapeutic inoculation of mice with vaccine formulations that have previously been shown to have a protective effect when given prior to challenge did not have any apparent effect on the course of the infection in the lungs. Interestingly, however, mice given the DNA-based vaccine and the CFP-based vaccine during the tuberculosis infection exhibited reductions in the bacterial load seen in the spleen. This is hard to interpret, since it could be due to a prevention of bacterial dissemination from the lungs to the spleen or could indicate better expression of immunity in the spleen itself, curtailing bacterial growth, or a combination of both.

The immunotherapeutic vaccination was given at a time when the animal was entering into a phase of chronic disease in the lungs, when the granulomatous response was well underway (13). The failure of T cells potentially boosted by the vaccines could simply be due to the fact that they could not focus on the granuloma structures, which by this time were relatively large, or alternatively could have happened because the bacteria themselves shut down metabolically and entered a latent stage in which relevant antigens such as Ag85 were not presented. Currently, we favor the first hypothesis, simply because bacteria in this chronic stage are still susceptible to isoniazid in vivo (8), indicating that mycolic acid biosynthesis, in which the Ag85 mycolyl transferase plays a key role (2), is continuing.

The observation that BCG given after challenge not only was ineffective but also seemed to trigger detrimental pathologic changes in the lungs was completely unexpected. The effect was marginal if BCG was given once but became more prolonged and severe if the vaccine was given twice or three times (at 15-day intervals). At this time, we can offer only speculations on this event. The first would be that BCG is boosting some type of immunity induced by the tuberculosis infection that causes direct local tissue damage, which would in turn cause the attraction of polymorphonuclear phagocytes. A second, perhaps more subtle, speculation is that the boosting response induced by the presence of BCG down-regulates or subverts the ordered generation of the granuloma. In this regard, we have seen a similar type of pathology developing in mice lacking γδ T cells (3) and have hypothesized (12) that such cells are involved in granuloma development with the minimum of tissue damage. Other mechanisms also could be subverted, such those mediated by IL-10 and transforming growth factor β in the down-regulation of acquired immunity (7, 17).

It has been suggested previously that BCG be used as a vector to carry *M. tuberculosis* genes no longer present in BCG or to express mammalian cytokines designed to boost immunity or used as an auxotrophic vaccine (9). While these are all promising avenues, the results here suggest caution in the use of BCG as an immunotherapeutic vaccine candidate. In fact, to date, only one vaccine candidate, a DNA vaccine derived from the hsp60 heat shock protein of *Mycobacterium leprae*, has been shown to have an immunotherapeutic vaccine effect (6). This effect is spectacular, with a thousandfold reduction in bacterial counts in both the spleen and lungs and complete prevention of reactivation of bacteria in a Cornell model system.

TABLE 1. Changes in bacterial load

Day	Postexposure vaccine	Value for organ: Lung CFU (mean $\log_{10}$)	Lung SEM	Spleen CFU (mean $\log_{10}$)	Spleen SEM	*P*
95	Saline control	5.2	0.2	4.2	0.5	
	BCG	5.4	0.4	3.9	0.1	
	DNA vector	5.2	0.3	4.5	0.4	
	DNA-Ag85	5.3	0.2	3.0	0.4	<0.01
	MPL-CFP	5.3	0.4	4.7	0.6	
	MPL–CFP–IL-2	5.4	0.3	3.4	0.1	0.059
	MPL	4.9	0.3	4.2	0.3	
150	Saline control	5.1	0.1	4.8	0.3	
	BCG	5.1	0.5	4.4	0.5	
	DNA vector	5.0	0.3	4.1	0.3	
	DNA-Ag85	5.0	0.1	3.8	0.4	<0.02
	MPL-CFP	5.2	0.4	4.5	0.1	
	MPL–CFP–IL-2	5.5	0.3	3.8	0.4	<0.01
	MPL	5.2	0.2	4.1	0.3	

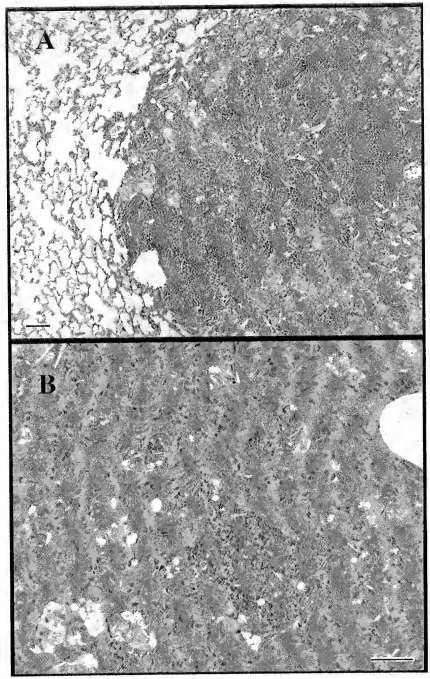

FIG. 2. (A) Large lymphocytic granuloma in the lungs of infected control mice (day 95). (B) Pyogranulomatous cellular infiltration creating abscess-like lesion in lungs of mice given three separate injections of BCG postaerosol (day 120). Bars, 100 μm.

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Therapy of tuberculosis in mice by DNA vaccination

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Mycobacterium tuberculosis **continues to kill about 3 million people every year[1], more than any other single infectious agent. This is attributed primarily to an inadequate immune response towards infecting bacteria, which suffer growth inhibition rather than death and subsequently multiply catastrophically. Although the bacillus Calmette–Guérin (BCG) vaccine is widely used, it has major limitations as a preventative measure[2]. In addition, effective treatment requires that patients take large doses of antibacterial drug combinations for at least 6 months after diagnosis[3], which is difficult to achieve in many parts of the world and is further restricted by the emergence of multidrug-resistant strains of *M. tuberculosis*. In these circumstances, immunotherapy to boost the efficiency of the immune system in infected patients could be a valuable adjunct to antibacterial chemotherapy[4]. Here we show in mice that DNA vaccines, initially designed to prevent infection, can also have a pronounced therapeutic action. In heavily infected mice, DNA vaccinations can switch the immune response from one that is relatively inefficient and gives bacterial stasis to one that kills bacteria. Application of such immunotherapy in conjunction with conventional chemotherapeutic antibacterial drugs might result in faster or more certain cure of the disease in humans.**

DNA vaccination protects mice against subsequent challenge with tuberculosis[5–7] by establishing a cellular immune response that is dominated by antigen-specific T lymphocytes that both produce interferon-γ (IFN-γ) and are cytotoxic towards infected cells (a type-1 cellular immune response). Both of these functions are probably required for maximally effective antimycobacterial immunity in mice[8] and in humans[9,10]. These responses are particularly favoured by DNA vaccines[11]. In contrast, antigen-specific T cells that produce interleukin-4 (IL-4) and are not cytotoxic (a type-2 cellular immune response) are abundant during infection with *M. tuberculosis*[12,13]. Such type-2 responses do not contribute to protection[8] and so a shift in the balance towards type-1 responses might be beneficial. Furthermore, because effective immunity to tuberculosis probably depends on possessing appropriate responses to multiple antigens, a broad shift in the phenotype of T cells

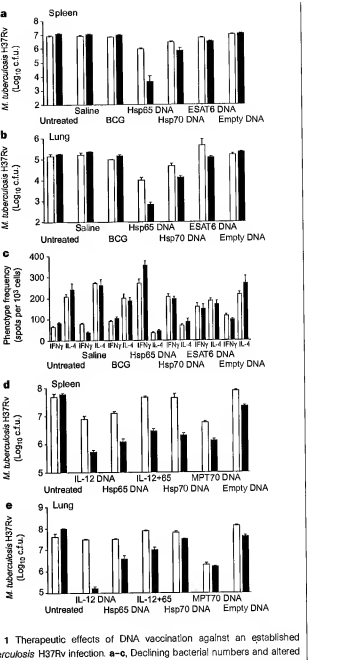

Figure 1 Therapeutic effects of DNA vaccination against an established *M. tuberculosis* H37Rv infection. **a–c**, Declining bacterial numbers and altered T-cell profiles after treatment with DNA encoding antigen Hsp65. Treatment commenced 8 weeks after intravenous injection of 5×10^5 live bacteria, when there were about 2×10^6 and 8×10^6 bacteria in spleens and lungs, respectively. Four intramuscular injections of plasmid DNA were given at 2-week intervals. The numbers of live bacteria in spleen (**a**) and in lung (**b**) 2 months (open bars) and 5 months (filled bars) after treatment commenced are shown as mean ± standard deviation (s.d.) ($n = 5$). Frequencies of lymph node cells (**c**) producing IFN-γ or IL-4 after 2 months (open bars) or at 5 months (solid bars) after treatment commenced were assayed by ELISPOT. Results are mean ± s.d. from triplicate determinations on cells pooled from groups of five mice. The effects of Hsp65 and Hsp70 DNAs were highly significant in both organs compared with empty plasmid or untreated controls at both time points (Student's *t*-tests, $P < 0.001$). **d**, **e**, Treatment with DNA expressing either antigen MPT70 or IL-12 also caused bacterial decline in spleen (**d**) and lung (**e**). Treatment commenced 8 weeks after intraperitoneal injection of 10^6 live bacteria when there were about 3×10^7 and 4×10^7 bacteria in spleen and lung, respectively. Counts of bacteria at 8 weeks (open bars) and 11 weeks (solid bars) after commencement of treatment are shown as mean ± s.d. ($n = 5$). The effects of IL-12, MPT70 and Hsp65 DNAs were highly significant in both organs at 11 weeks compared with either empty DNA or untreated controls (Student's *t*-tests, $P < 0.001$).

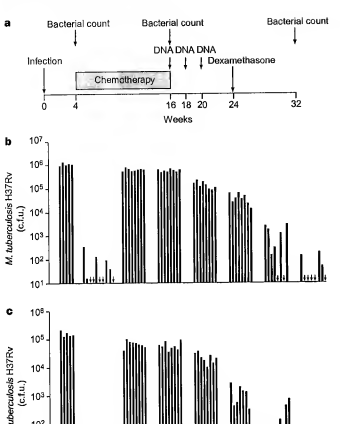

Figure 2 Elimination of residual *M. tuberculosis* by DNA vaccination after chemotherapy. Mice were given isoniazid plus pyrazinamide chemotherapy in the diet from week 4 of infection to week 16. Hsp65 DNA vaccine was given one, two or three times at 2-week intervals after completion of chemotherapy, followed by immunosuppression by dexamethasone treatment at 24 weeks. **a**, The schedule of treatments and sampling. **b**, **c**, Bacterial counts from the organs of individual mice; arrows indicate organs with counts below the level of detection. At week 16, only a few bacteria were detected in the spleens (**a**) of 5/10 mice and lungs (**b**) of 2/10 mice; no bacteria were detected in the remainder. Bacterial counts at 32 weeks are shown for groups of eight mice that received one, two or three doses of Hsp65 DNA, three doses of empty plasmid or saline, or a single injection of BCG at week 16. At week 32, only a few bacteria were detected in the spleens of 3/8 mice and lungs of 0/8 mice that had received three doses of the DNA vaccine before dexamethasone.

responding to many different antigens might be needed for maximum therapeutic effect.

DNA vaccination may be particularly suited to achieve such a shift because of an immunostimulatory 'adjuvant' effect of plasmid DNA itself[14]. Indeed, prophylactic DNA vaccination against tuberculosis increased the proportion of spleen T cells that expressed an activated (CD44hi) phenotype (associated with the type-1 response) from about 12 to 40%, even though less than one in a thousand were specific for the immunizing antigen (a heat-shock protein with a relative molecular mass of 65,000 (*M*, 65K): antigen Hsp65)[8]. Therapeutic effects of DNA vaccination against *Mycoplasma pulmonis* have also been reported[15,16]. Therefore we tested whether DNA vaccination could have a beneficial effect and enhance the type-1 response during the course of tuberculosis.

An infection initiated by intravenous injection of virulent *M. tuberculosis* H37Rv was allowed to develop for 8 weeks, during which time the numbers of bacteria in the internal organs increased slowly by about four-fold. Mice were then given a series of four doses of plasmid DNA intramuscularly at 2-week intervals. The numbers of live bacteria in spleen and lungs declined rapidly 2 months and 5 months after the first dose of DNA when the plasmid expressed antigen Hsp65 (Fig. 1a, b). Much smaller effects were obtained with plasmid expressing other mycobacterial antigens (the 70K heat-shock protein or the 6K early secretory antigen: Hsp70, ESAT6) and this parallels the lower efficacy of these antigens in prophylactic DNA vaccination[17]. There was virtually no change induced with plasmid that contained no insert, or by a single dose of the standard BCG vaccine, compared with untreated or saline-treated control mice. To test if the therapeutic vaccination had modified T-cell priming we estimated the frequencies of T cells that typified type-1 or type-2 immune responses in the lymph nodes of treated and untreated mice. The therapeutic effect of DNA vaccination was indeed associated with a switch from a predominantly type-2 to a predominantly type-1 response (Fig. 1c). DNA-vaccine therapy was equally effective against an established infection with a clinical isolate of *M. tuberculosis* (CLS52) that is resistant to isoniazid, one of the main chemotherapeutic drugs, and an identical change in T-cell phenotypes was observed (data not shown).

This unexpected efficacy of DNA-vaccine therapy was confirmed in an experiment in which a higher dose of bacteria was given and the bacteria attained much higher levels of infection (Fig. 1d, e). Although the magnitude of the observed therapeutic effect of plasmid expressing Hsp65 was somewhat less, the experiment had to be curtailed because the control mice that were untreated or received empty plasmid began to show clinical signs of advanced tuberculosis. In this experiment, either a plasmid expressing a 22K mycobacterial secretory antigen (MPT70) that may be upregulated in infection[18,19], or a plasmid expressing interleukin-12 (IL-12; see below), was at least as beneficial as a plasmid expressing Hsp65.

Much of the adjuvant effect of DNA vaccines is due to their content of non-methylated CpG sequences that induce IL-12 secretion by cells engaged in antigen presentation[14]. IL-12 selectively promotes the acquisition of a type-1 phenotype and of cytotoxicity during priming and maturation of adjacent lymphocytes, irrespective of their antigen specificity[20]. Furthermore, this cytokine is crucial in the development of protective immunity against tuberculosis[6,21–23]. Therefore we also tested the therapeutic effect of a plasmid that expressed IL-12 instead of a mycobacterial antigen. This plasmid caused the greatest reductions in bacterial numbers by 11 weeks (Student's *t*-tests, $P < 0.001$), although co-delivery of plasmids expressing IL-12 and Hsp65 as a 50:50 mixture resulted in antagonism and reduced therapeutic efficacy. The basis of such interactions between plasmids and the identities of the therapeutic T cells elicited by plasmids expressing either antigens or IL-12 are the subject of further investigation.

When modern chemotherapy fails, it most often does so as a result of re-growth of fully drug-sensitive bacteria that have persisted in a non-replicating and physiologically drug-resistant form[3,24]. We tested whether the addition of DNA vaccination at the end of chemotherapy could reduce this problem. Three intramuscular doses of Hsp65 DNA vaccine appeared to eliminate residual bacteria from a proportion of the mice when the DNA was given after chemotherapy (Fig. 2); injections of immunosuppressive corticosteroid were unable to reactivate bacterial growth, indicating a sterilizing effect[25]. The proportion of mice with spleens or lungs that appeared to be sterile increased significantly as the number of DNA doses was increased (trend χ^2 test, $P < 0.001$).

In the models of tuberculosis tested here, infection was established by a large systemic dose of bacteria. This not only presented a severe challenge for therapy, but it also reflected the usual aetiology of human tuberculosis. Blood-borne dissemination of bacteria from the primary pulmonary focus of infection is needed to establish the secondary foci that are the main cause of disease[26]. However, mice have a relatively high innate resistance to tuberculosis and natural

transmission of most human tuberculosis is airborne, by inhalation of small numbers of bacteria. Consequently, it will be appropriate to evaluate immunotherapy in other more susceptible animal models and after airborne infection. In addition, the effect of giving chemotherapy and immunotherapy simultaneously should be assessed, as this would be most useful clinically. Nevertheless, the present findings strengthen the case for evaluating immunotherapy as an adjunct to chemotherapy[4]. Furthermore, persistence of the bacteria can occur after natural resolution of the initial infection in humans. Bacterial re-growth many years later when the immune system declines accounts for most of the tuberculosis found in developed countries[27]. We can speculate therefore that similar vaccines used prophylactically and therapeutically might be able to both prevent establishment of this persistent state and eliminate it if it is already established.

Note added in proof: Results of preliminary experiments in which Hsp65 DNA therapy was given 8 weeks after aerosol infection of either mice or guinea pigs have indicated substantial therapeutic benefits in these models also, decreasing c.f.u. and prolonging survival respectively. □

Methods

Therapy of established infection by DNA vaccination. Female Balb/c mice aged 6–8 weeks were infected by either intravenous or intraperitoneal injection with *M. tuberculosis* H37Rv. DNA vaccination was initiated after 8 weeks and was done by injection of 50 μg of plasmid DNA in 50 μl saline into the quadriceps muscle of each hind leg, on four occasions at 2-week intervals. When plasmid mixtures were given, they were injected using the same total amount of DNA per dose (50 μg). Control mice received empty plasmid or saline injections only or a single subcutaneous injection of 10^5 live BCG in 50 μl saline at 8 weeks. Mice were killed at intervals and bacteria in internal organs were counted as colony forming units (c.f.u.) on 7H11 medium[4].

Plasmids. DNA sequences encoding *M. leprae* Hsp65, *M. tuberculosis* Hsp70 or *M. tuberculosis* ESAT6 antigens were cloned into pcDNA3 (Invitrogen)[17]. For the experiment shown in Fig. 1d, e, we used pCMV4. This differs from pcDNA3 by containing, in addition to the promoter of the cytomegalovirus (CMV) immediate early gene, the first intron (IE-1), cloned from hCMV strain 169 as a *Hind*III-terminated 0.9-kilobase product by a polymerase chain reaction; ref. 28). Inclusion of the intron can give higher levels of antigen expression[29]. Plasmid expressing murine IL-12 (pIRES muIL-12) was constructed on a backbone that was similar to pCMV4 and used an internal ribosome entry site to express both the p35 and p40 subunits from the CMV IE-1 promoter/enhancer[30].

T-cell frequencies. Lymphocytes were obtained from the pooled inguinal and mesenteric lymph nodes. Plastic- and nylon-adherent cells were removed and the frequencies of cells producing IFN-γ (type-1 phenotype) or producing IL-4 (type-2 phenotype) in the presence of phorbolmyristic acid (10 ng ml^{-1}) and monoclonal antibody against CD3 (YCD3-1, 50 ng ml^{-1}; Gibco-BRL) were determined by limiting dilution ELISPOT analysis[8].

Elimination of persistent bacteria by DNA vaccination. Four weeks after the initiation of infection in Balb/c mice by intravenous injection of 5.4×10^6 live *M. tuberculosis* H37Rv, mice were placed on a standard diet supplemented with isoniazid (25 mg kg^{-1}) and pyrazinamide (1,000 mg kg^{-1}) for 12 weeks and the decline in c.f.u. in spleens and lungs was monitored. Mice were then given one, two or three intramuscular doses of 100 μg Hsp65 DNA vaccine at 2-week intervals, three doses of empty plasmid, or a single subcutaneous dose of live BCG. Eight weeks after the first dose of DNA, three subcutaneous injections of dexamethasone (6 mg kg^{-1}) were given 2 days apart and bacterial c.f.u. in lungs and spleens were measured after a further 8 weeks.

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ovo/svb integrates Wingless and DER pathways to control epidermis differentiation

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In *Drosophila*, as in mammals, epidermal differentiation is controlled by signalling cascades[1] that include Wnt proteins[2,3] and the *ovo/shavenbaby* (*svb*) family of zinc-finger transcription factors[4–6]. *Ovo/svb* is a complex gene with two genetic functions corresponding to separate control regions: *ovo* is required for female germline development and *svb* for epidermal morphogenesis[7,8]. In the *Drosophila* embryo, the ventral epidermis consists of the segmental alternance of two major cell types that produce either naked cuticle or cytoplasmic extrusions known as denticles. Wingless signalling specifies smooth cells that produce naked cuticle[9], whereas the activation of the *Drosophila* epidermal growth factor (EGF) receptor (DER) leads to the production of denticles[10]. Here we show that expression of the *ovo/svb* gene controls the choice between these cell fates. We find that *svb* is a key selector gene that, cell autonomously, directs cytoskeletal

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Pulmonary Necrosis Resulting from DNA Vaccination against Tuberculosis

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The use of DNA constructs encoding mycobacterial proteins is a promising new approach to vaccination against tuberculosis. A DNA vaccine encoding the hsp60 molecule of *Mycobacterium leprae* has previously been shown to protect against intravenous infection of mice with *Mycobacterium tuberculosis* in both the prophylactic and immunotherapeutic modes. It is shown here, however, that this vaccine was not effective in a more realistic aerosol infection model or in a model of latent tuberculosis in the lungs. Moreover, when given in an immunotherapeutic model the immunized mice developed classical Koch reactions characterized by multifocal discrete regions of cellular necrosis throughout the lung granulomas. Similar and equally severe reactions were seen in mice given a vaccine with DNA coding for the Ag85 antigen of *M. tuberculosis*. This previously unanticipated safety problem indicates that DNA vaccines should be used with caution in individuals who may have already been exposed to tuberculosis.

Disease caused by *Mycobacterium tuberculosis* is the leading cause of death from an infectious disease. Currently, it is estimated that over 2 million people die from tuberculosis yearly and that 8 million people contract the disease (5). Individuals who have been exposed to the bacillus but may have controlled it in the form of a latent infection, and who therefore are at risk of reactivation disease, may number in the hundreds of millions (6).

There has been a gradual realization that the existing vaccine against tuberculosis, *Mycobacterium bovis* BCG, loses its effectiveness in individuals as they pass the teenage years (3, 21), and in certain clinical trials its overall effectiveness has been zero (14). As a result there is now a concerted effort to try to identify more effective vaccines against tuberculosis, using well-characterized animal models for initial screening (16).

To date, the most effective vaccine used in mouse studies is a DNA vaccine encoding the 65-kDa heat shock protein of *Mycobacterium leprae* (hsp60/lep). Mice immunized with this DNA vaccine have shown 2- to 3-log reductions in bacterial load after intravenous challenge with virulent *M. tuberculosis*, and the vaccine is equally protective if given prophylactically, as an immunotherapeutic vaccine, or in a Cornell type model in which bacteria are eliminated by chemotherapy, leaving only bacilli that are in a latent or dormant form (12).

In the study presented here the hsp60/lep vaccine was tested using the more realistic pulmonary infection model in the mouse, in which the animal is exposed to a small challenge dose by aerosol exposure. In this model the hsp60/lep DNA vaccine was ineffective in both the prophylactic and Cornell modes. Moreover, this vaccine, as well as the previous described highly protective Ag85/TB DNA vaccine (1, 8, 25), both induced progressively severe pulmonary necrosis in an immunotherapeutic vaccination model, especially when given to a susceptible mouse strain. While this class of DNA vaccines appears to be completely safe in terms of inoculation into naïve individuals, the data presented here suggest that if given unknowingly to individuals already exposed to tuberculosis, there may be a risk of exacerbating disease or triggering reactivation of latent tuberculosis. This previously unanticipated problem thus raises a significant safety issue.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 and BALB/c mice, 6 to 8 weeks old, were purchased from the Jackson Laboratories, Bar Harbor, Maine. The mice were maintained under barrier conditions in the biosafety level 3 biohazard facility at Colorado State University. They were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments.

Vaccinations. The DNA vaccine encoding the hsp60 protein antigen of *M. leprae* was constructed using the plasmid vector pcDNA3 (12). Ag85/TB DNA was prepared and used as previously described (1, 8). Mice received injections intramuscularly (i.m.) three times at 3-week intervals with 50 μg of hsp60-DNA per quadriceps muscle using a 30-gauge needle. As negative controls, mice received injections with control plasmid DNA or with saline. As a positive control, mice received subcutaneous injections with BCG at 10^6 CFU in 200 μl of sterile 1× phosphate-buffered saline (single inoculation).

Animal challenge studies. In all experiments, mice were challenged by low-dose aerosol exposure with *M. tuberculosis* strain H37Rv using a Glas-Col (Terre Haute, Ind.) aerosol generator calibrated to deliver 50 to 100 bacteria into the lungs. Bacterial counts in the lungs were determined by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar and counting the CFU following 3 weeks incubation of at 37°C. In the prophylactic model, mice were challenged 4 weeks after the last DNA injection, and lungs were harvested for bacterial counts and histological analysis 4 and 10 weeks following aerosol infection. In the Cornell model, mice were aerogenically infected with *M. tuberculosis* 4 weeks prior to receiving isoniazid (Sigma) in their drinking water at 100 mg/ml for 60 days. Immediately following drug treatment, the mice received i.m. injections with hsp60-DNA three times as described above. Three weeks after the last DNA injection, mice received intraperitoneal injections with 200 μl of dexamethasone (Sigma) at 6 mg/kg of body weight every 2 days for 8 weeks. Lungs were harvested 18 days later for bacterial counts and histological analysis. In the therapeutic model, mice received the first hsp60-DNA injection 8.5 weeks

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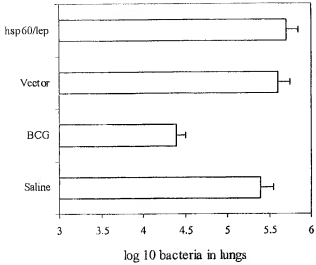

FIG. 1. Prophylactic vaccination in C57BL/6 mice. Animals were given DNA or control vector DNA three times and then rested for 1 month prior to low-dose aerosol challenge with *M. tuberculosis* H37Rv. Controls received injections of saline or a single dose of BCG. Data shown are the mean numbers of bacteria recovered from the lungs 30 days after aerosol exposure ($n = 5$) (error bars, standard errors of the means). Only mice receiving BCG were protected against the challenge infection ($P > 0.01$).

after the aerosol infection. Lungs were harvested for bacterial counts and histological analysis 4 and 7 weeks after the last DNA injection.

Histological analysis. The middle right lung lobe was obtained from each mouse and immediately inflated with and stored in 10% formal saline. Tissues were processed routinely and sectioned for light microscopy such that the maximum surface area of each lobe could be viewed. Sections were then stained with hematoxylin and eosin. Slides were examined by a veterinary pathologist with no prior knowledge of the experimental groups and were subjectively graded for both quality and quantity of cellular accumulation.

RESULTS

Prophylactic vaccination studies. In a first series of experiments BALB/c were immunized either three or four times with hsp60/lep DNA prior to challenge by aerosol with *M. tuberculosis* strain H37Rv. In these studies no evidence of protection was seen, and so the experiments were repeated using an intrinsically more resistant strain, C57BL/6, in which previously good results have been obtained with other DNA vaccines (1). However, here again no reduction in the bacterial load in the lungs was observed (Fig. 1). Because of previously observed (24) detrimental pathology using a similar vaccine (hsp60/TB DNA) the lungs of these mice were carefully examined. In sections of lungs examined 30 days after challenge infection the overall pathology observed was similar in the vaccinated mice and in saline controls, with both showing widespread granulomatous inflammation (Fig. 2A and B). In sections examined 80 days later the aggregates of lymphocytes and macrophages were large and appeared to be much more extensive in the mice receiving the DNA vaccine, suggesting an increased cellular influx (Fig. 2C and D).

Postchemotherapeutic vaccination studies. A second series of studies were performed in both BALB/c and C57BL/6 mice using the so-called Cornell protocol (19), in which infected mice are given chemotherapy to reduce the bacterial load to below detectable levels, after which steroid immunosuppression is given to try to reactivate residual surviving bacteria. Following the protocol previously described for the hsp60/lep

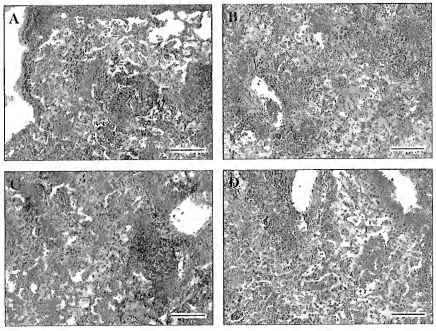

FIG. 2. Photomicrographs of lungs of C57BL/6 mice harvested 30 days (A and B) or 80 days (C and D) after aerosol exposure to *M. tuberculosis*. Mice received prophylactic vaccination with hsp60/lep (A and C) or saline (B and D). Alveoli contain large numbers of macrophages and lower numbers of lymphocytes, but there is no appreciable neutrophil infiltration or necrosis. At day 80 perivascular accumulations of lymphocytes are seen; these were particularly prominent in the hsp60/lep vaccinated animals.

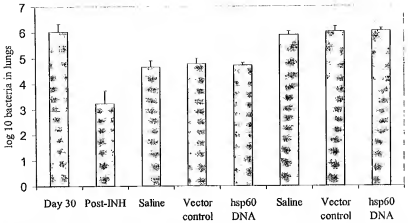

FIG. 3. Effect of hsp60/lep vaccine in the modified Cornell model. Four weeks after exposure to low-dose aerosol challenge with *M. tuberculosis* H37Rv, the bacterial load was assessed (day 30 counts), and then the remaining mice were treated with isoniazid for 60 days, after which the reduction in bacterial load was assessed (Post-INH). Mice were then given three vaccinations with the hsp60/lep DNA or control injections. Thirty days after the final vaccination the bacterial load was determined, and it was then determined again after 8 weeks of treatment with dexamethasone. In each case the mean value is shown ($n = 5$) (error bars, standard errors of the means).

DNA vaccine (12) inoculation with this vaccine was given prior to steroid reactivation. Despite the large number of mice used, only a small percentage of animals showed evidence of reactivation (range of one to three mice having a detectable bacterial colony in their lungs per group of 15 animals), which did not permit analysis of any potential differences between groups. In view of this, we attempted to address this problem by repeating the experiment using a deliberately less effective chemotherapeutic regimen, isoniazid alone for 60 days, that we knew from previous experience will reduce the bacterial load considerably but would not completely eliminate the bacterial load in the infected mice, at least not to the extent that this could be determined bacteriologically. In this study (Fig. 3) isoniazid treatment reduced the bacterial load by 3 logs in the lungs of the infected mice. Groups of mice were then given hsp60/lep DNA, vector alone, or saline, and the bacterial load was assessed 30 days after the third vaccination. As shown, none of these materials had any effect on the regrowth seen in the bacterial load in the lungs. Moreover, since these values were still relatively low, a second group of treated mice were given 8 weeks of treatment with daily doses of dexamethasone to see if this altered any of the groups. As shown, the steroid therapy exacerbated the bacterial load to a similar degree in all three groups, measured 18 days after cessation of 8 weeks of dexamethasone treatment. These data thus indicate that the DNA vaccine had no effect, even when the bacterial load was very low.

Immunotherapeutic vaccination studies. In a third series of experiments, we examined the ability of the hsp60/lep and the Ag85 DNA vaccines to reduce the bacterial load in the lungs if given after the mouse had already been infected with *M. tuberculosis*. The first set of studies were performed in BALB/c mice that are relatively susceptible to aerosol infection. The protocol was designed to follow the bacterial load starting 1 month after the final DNA vaccine administration, but a few days after this vaccination was completed many of the mice began to die, and others appeared to be in severe distress and were therefore euthanized in accordance with our animal usage approval protocol.

The lungs from these mice were then examined and animals receiving saline or control DNA had expected discrete granulomas consistently seen in mice 4 to 5 months after aerosol exposure. These lesions contained mixtures of lymphocytes and macrophages, with some of the latter cells appearing foamy and being gradually surrounded by increasing fibrosis (Fig. 4A). In contrast, granulomas in mice receiving hsp60/lep DNA were very big (as much as 10-fold larger than those in controls) and contained multiple areas of destructive necrosis throughout these structures (Fig. 4B). Higher magnification of these areas revealed foci consisting of groups of dead macrophages, often containing large clumps of acid fast staining bacteria (Fig. 4C and D). Similar areas of necrotic foci were seen in mice given the Ag85 DNA vaccine (data not shown).

Again, given these unexpected results, the studies were repeated using C57BL/6 mice, and with the protocol modified to reduce the vaccine frequency given from four to three. In these subsequent experiments no deaths were observed in any of the animals, but no reductions in the bacterial load were seen 30 days after the third inoculation with the DNA vaccine (Fig. 5). Examination of the lungs of these animals at this time point again revealed large areas of degenerative pathology in the DNA vaccinated mice (Fig. 6). Interestingly, a similar pattern was seen in the lungs of mice 100 days after the final vaccination, but in many sections the pathology seemed less pronounced, suggesting that some degree of tissue repair had taken place (Fig. 7).

DISCUSSION

The results of this study show that a plasmid DNA vaccine (hsp60/lep) encoding the hsp60 molecule of *M. leprae* that has been previously shown to be highly effective against intravenous or intraperitoneal inoculation with virulent *M. tuberculo-*

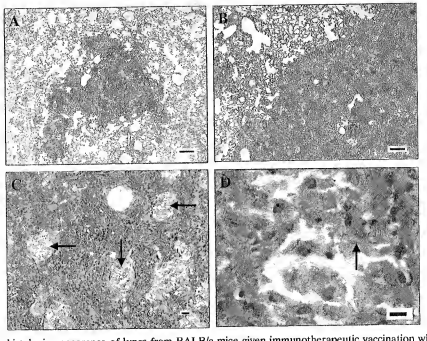

FIG. 4. Representative histologic appearance of lungs from BALB/c mice given immunotherapeutic vaccination with hsp60/lep. (A) In mice given saline only small granulomatous foci were seen in the lungs of these animals 130 days after low-dose aerosol challenge with *M. tuberculosis* H37Rv. Perivascular aggregates of lymphocytes can be seen extending into the adjacent parenchyma, which is variably surrounded by macrophages. Size bar = 100 μm. (B) In mice given hsp60/lep DNA the lesions are very large (size bar = 100 μm) and show evidence of extensive tissue destruction. There is a sharp line of demarcation with normal lung parenchyma, and alveoli are filled with infiltrates of mononuclear cells and some neutrophils. There is loss of alveolar wall detail, with multifocal necrosis. (C) At a higher power of magnification (size bar = 10 μm), these multiple necrotic foci are characterized by fibrillar acidophilic cellular debris with clear clefts that represent extracellular cytoplasmic lipid deposition (arrows). These foci contain a mixture of normal to completely degenerate macrophages. (D) Acid-fast staining revealed multiple bacilli (example indicated with an arrow) visible within the cytoplasm of foamy macrophages as well as in the extracellular space of necrotic foci. Such bacilli were only rarely seen in lesions of control mice at this stage of the infection.

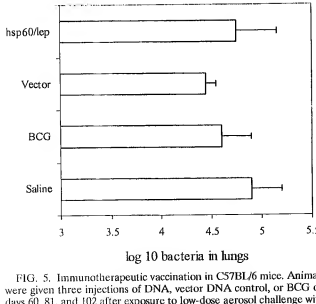

FIG. 5. Immunotherapeutic vaccination in C57BL/6 mice. Animals were given three injections of DNA, vector DNA control, or BCG on days 60, 81, and 102 after exposure to low-dose aerosol challenge with *M. tuberculosis* H37Rv. The data shown are the mean numbers of bacteria recovered from the lungs after a further 30 days ($n = 5$) (error bars, standard errors of the means). No protection was seen in any groups at this time, or after a further seventy days (data not shown).

sis H37Rv failed to protect mice if the infection was given by the more realistic pulmonary route.

The previous observation (12) that the hsp60/lep had such a substantial protective effect in mice given this DNA construct as a prophylactic vaccine against intravenous challenge is of great interest, since it implies that the bacterium produces large amounts of this antigen early during the course of the infection. In contrast, it is very interesting that culture filtrates obtained from bacteria growing progressively in nutrient medium in vitro contain only trace amounts of hsp60, and such cultures only begin to produce significant amounts of hsp60 if deprived of nutrients or exposed to low oxygen tensions. Whatever the explanation, the data in the present study suggest that alveolar and/or local resident macrophages in the lungs do not present the hsp60 antigen, or the bacillus does not produce it perhaps because of the very high local oxygen tension. Alternatively, hsp60 antigen may be expressed by infected macrophages in the lungs, but due to the influx of other such (uninfected) cells giving rise to the characteristic epithelioid macrophage granuloma in which the great majority of the cells do not contain live bacteria, immune T cells fail to reach those cells actively infected. This situation may arise because, unlike secreted proteins which are recognized very early on (15), the kinetics of presentation of hsp60 may be slower as a result of bacilli only starting to produce this protein when exposed to host defenses, such as reactive oxygen and nitrogen radicals (4, 7). Of course, if this is the correct explanation, then it implies

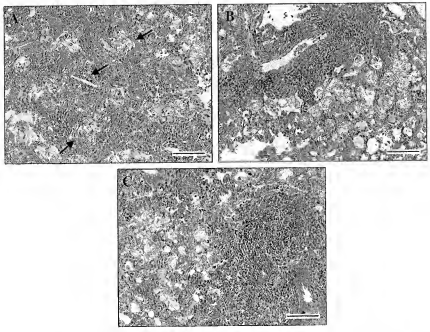

FIG. 6. Histologic appearance of lungs of C57BL/6 mice 30 days after the third immunotherapeutic vaccination with hsp60/lep. (A) Alveoli and interalveolar septae are effaced by infiltrates of macrophages, some of which have abundant foamy cytoplasm. There are random foci of necrosis containing karyorhectic and cellular debris with intralesional aggregates of neutrophils (indicated by an arrow). Mice given saline (B) or DNA vector control (C) have prominent perivascular accumulations of lymphocytes and intra-alveolar macrophages but no necrosis or neutrophilic infiltration.

that the vaccine would almost certainly be ineffective under clinical conditions. Having said that, this does not diminish the importance of trying the DNA class of vaccines under such conditions, especially considering that nearly a century has passed since the BCG vaccine was first developed as a potential defense against tuberculosis, and the field has failed since then to provide a valid alternative.

Our failure to observe no more than minimal reactivation in our initial study using the Cornell model was almost certainly due to two reasons. Firstly, the combination chemotherapy

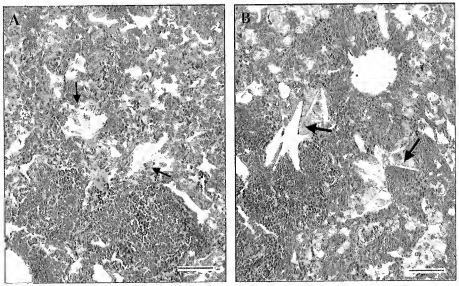

FIG. 7. Histologic appearance of lungs one hundred days after immunotherapeutic vaccination with hsp60/lep DNA. Foci of necrosis contain debris (small arrows) and clefts of accumulated cholesterol (large arrows). Alveoli contain macrophages with abundant foamy cytoplasm, and aggregates of lymphocytes are prominent. Overall, the appearances of lungs from vaccinated (A) and saline controls (B) were similar.

protocol is extremely potent in the mouse, and 4 months of treatment will reduce the number of potentially reactivating mice to a low percentage (in this study the highest reactivation observed was 3 out of 15 mice) as well as increasing the number of mice needed per group to preserve statistical power. Secondly, although most if not all mice can be induced to reactivate if sufficient steroid treatment is subsequently given, this treatment generally has to be given for an extended period of time to be fully effective. Whereas only three injections of dexamethasone resulted in full reactivation of all control animals and more than 10^4 bacterial colonies being detected in the lungs of each of these mice in the study described by Lowrie et al. (12), in our own experience (2) at least seven injections of the more potent hydrocortisone only gave rise to partial reactivation of mice and bacterial counts of <100. In a more recent, very comprehensive analysis of this type of model, a study by Scanga et al. (19) took even longer to induce reactivation in mice given a similar chemotherapeutic regimen, and again the recovery of bacterial colonies was very low (a mean of 70).

To try to address this we used a modified protocol that we knew would not completely reduce the bacterial load to undetectable levels. We reasoned that although it is was probable that this protocol would not drive all bacteria into a truly latent state, as some proponents of the Cornell model seem to believe, it would still he a fair test of the capacity of the hsp60/lep DNA vaccine to reduce or eliminate the presence of the remaining bacilli. However, as shown above we were unable to find any evidence that the DNA vaccine could reduce the bacterial load in the lungs.

Administration of both the hsp60/lep and the Ag85/TB DNA as immunotherapeutic vaccines to BALB/c mice already in a state of chronic disease resulted in death of the animals within a few days of receiving the final i.m. inoculation. Autopsy of the few remaining mice in both cases revealed multiple foci of small circular areas of necrotic tissue damage throughout the granulomatous structures, with similar damage seen in mice receiving either vaccine. Moreover, in both cases lesions were noticeably much larger than in controls, suggesting a florid lymphocytic influx induced by the vaccines. A repeat of this study in resistant C57BL/6 mice in which the DNA immunization frequency was reduced did not result in fatalities, but the vaccination had no effect on the bacterial load in the lungs. Some lung damage was observed in the DNA-vaccinated mice, but interestingly this did not appear to have further progressed in animals examined 100 days later, possibly indicating that some degree of tissue repair had taken place.

The results shown here, as well as very similar recent results by others (17) attempting DNA vaccination in an immunotherapeutic or Cornell style model, clearly show that the generation of protection against an active infection using vaccines in an immunotherapeutic manner creates a major challenge. Since the vaccine would be given at a distant site, T cells generated as a result would have to able to rapidly home to the site of infection and have the necessary molecules to effectively invade the granulomatous tissue (which by that time could be in an advanced state of formation). Moreover, by that time the increasing local fibrosis and micronecrosis that is a primary facet (18) of chronic tuberculosis in the mouse lung would almost certainly be a further hindrance. Having said that, the data presented here seem to suggest that the hsp60/lep induced a considerable lymphocytic cellular influx, which was seen in both the prophylactic and immunotherapeutic models.

Collectively, these data indicate that the previously described hsp60/lep DNA vaccine, so effective in intravenous infection models (12), lacks the capacity to protect mice against pulmonary tuberculosis when delivered by the more realistic acrogenic route. In addition, a previously unanticipated observation concerned the apparently accelerated development of widespread necrotic lesions in the lungs of such animals, especially if the vaccine was given in an immunotherapeutic mode.

Two findings should be strongly stressed here. The first is that the common finding that both DNA vaccines tested here, as well as our previous experience with an hsp60/TB DNA construct (24), seems to imply that the pathological effects observed appear to be a general phenomenon of DNA vaccines encoding immunogenic mycobacterial antigens and are clearly not limitcd to vaccines encoding the heat shock chaperonin proteins. The frequency of immunization is also an important factor, since no adverse effects were seen until the mice had received at least three injections. In a previous study in which Ag85 DNA was given just twice we saw no protection, but we also no evidence of untoward pathological effects (23). Additionally, the relative resistance of the host seems to be important, given the lack of mortality seen in the C57BL/6 mice. It is interesting that it has previously been demonstrated (9) that BALB/c mice infected with *M. tuberculosis* react much more strongly to hsp60 protein than mice on the B6 background.

The second finding to be stressed is that all of the DNA vaccines tested to date in this laboratory appear to be completely safe in the context of the inoculation procedure itself. It is only after the animal has been exposed to *M. tuberculosis* infection that these pathological effects become evident, and only then if they are given repetitively. Moreover, since these pulmonary lesions take time to develop this undoubtedly explains why they were not noted in the intravenous challenge model described by Lowrie et al. (12), in which no histological data appear to have been collected. Nevertheless, these findings raise a safety issue and may imply that DNA vaccines given to individuals who unknowingly already have an undetected lesion in their lungs containing chronic or latent bacilli run the risk of inducing necrotic reactivation. In this regard, there are several DNA vaccines under test that have given good results to date (16) including ESAT-6, MPT64, and others, and it is unknown if such potential vaccines would also induce these problems.

The potential use of hsp60, at least in the context of a protein vaccine, has had a checkered history. It is clearly highly immunogenic, especially if bacteria are heat killed as a result of which their last physiological response is to make large amounts of this material (10). Inoculated in this manner there is a potent TH2 type response, resulting in large amounts of antibody production, but also evidence of a strong cytotoxic T lymphocyte response (28), an observation also seen in terms of the response to the DNA vaccine. However, their promise as a vaccine was initially discounted by studies over 15 years ago by Wiegeshaus and Smith, who failed to protect guinea pigs

against aerosol infection with virulent *M. tuberculosis* using a recombinant vaccine (27). As a result, the use of this highly conserved heat shock protein, with its attendant autoimmune potential counteracting any potential benefits as an antimicrobial vaccine (26), remains debatable.

In this regard, the multiple pockets of necrosis seen in the lungs of the DNA-vaccinated mice in the immunotherapeutic studies are reminiscent of the famous Koch reaction in which Koch found that immunization of mice with a suspension of mycobacterial antigens triggered necrosis in preexisting lesions in the lungs of these animals (11). A potential explanation for these observations is the generation of potent cytotoxic T lymphocyte CD8 responses induced by the hsp60/lep DNA vaccine, as Silva and colleagues have demonstrated (20).

It is also potentially possible that BCG vaccination can itself induce such reactions. In mice given BCG in the immunotherapeutic mode we previously observed evidence for a pyogenic response in the lungs, suggesting lung damage (23). It is therefore interesting to speculate that in the south India BCG vaccine trial (22), administration of BCG to individuals already latently infected with tuberculosis may have induced such Koch reactions, thus explaining the higher incidence of eventual tuberculosis in the vaccinated group compared to the unvaccinated controls. Even today the basis of Koch's observation is not known precisely (13), and so we have begun immunotherapeutic vaccination experiments using various models with mice with gene disruptions to try to identify if it is indeed CD8 T cells, or perhaps others, that underlie such potentially fatal responses, and which thus act adversely against an otherwise useful vaccination strategy.

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United States Patent [19]

Rook et al.

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[54] **IMMUNO-THERAPEUTIC COMPOSITION OF KILLED CELLS FROM MYCOBACTERIUM VACCAE**

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[58] **Field of Search** 424/88, 92, 93; 435/68, 435/863, 822

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[57] **ABSTRACT**

"Immuno-therapeutic agents of killed cells prepared from *Mycobacterium Vaccae* that are useful in the treatment of mycobacterial disease, especially tuberculosis or leprosy, in particular as an adjuvant to chemotherapy".

11 Claims, No Drawings

IMMUNO-THERAPEUTIC COMPOSITION OF KILLED CELLS FROM MYCOBACTERIUM VACCAE

This invention relates to immunotherapeutic agents useful in the immunotherapy of mycobacterial disease, especially tuberculosis and leprosy.

The eradication of mycobacterial diseases such as tuberculosis and leprosy by effective treatment is still a primary objective particularly in disease endemic areas such as third world countries of Asia, Africa and South East Asia. Modern drug treatment of these diseases consists of chemotherapy with, for example, rifampicin and isoniazid in the case of tuberculosis and clofazimine and sulphones in the case of leprosy.

Chemotherapy, though effective in killing rapidly metabolising bacilli, is very slow to eliminate "persisters", and this necessitates continuation of treatment for 9 months to a year in the case of tuberculosis, and 5 years or more in the case of leprosy. 'Persisters' are metabolically inactive microorganisms which can survive long exposure to a drug, only becoming susceptible when they start to multiply.

We have now found that the mycobacterium, *M. vaccae*, is especially effective for the immunotherapy of mycobacterial disease, especially tuberculosis and leprosy. Experiments have shown that suspensions containing killed *M. vaccae* in excess of 10^8 microorganisms per ml of diluent can be effective in eliminating "persisters" within a short period of time, usually 1 or 2 months. In addition, vaccines based on *M. vaccae* are easy to manufacture at low cost since *M. vaccae* can be cultivated in simple media, unlike some other species of mycobacteria, for example *M. leprae*, which can only be cultivated in armadillo tissues which are expensive and not easily obtainable.

The present invention therefore provides an immunotherapeutic agent comprising antigenic material derived from *Mycobacterium vaccae*. The antigenic material is conveniently, and therefore preferably, dead cells of *M. vaccae*, e.g. cells which have been killed by irradiation. The immunotherapeutic agent normally comprises more than 10^8 microorganisms per ml of diluent, and preferably from 10^8 to 10^{11} killed *M. vaccae* microorganisms per ml of diluent. The invention includes within its scope antigenic material from *M. vaccae* for use in therapy in the treatment of mycobacterial disease, e.g. tuberculosis or leprosy, preferably as an adjunct to chemotherapy.

The diluent may be pyrogen-free saline for injection alone, or a borate buffer of pH 8.0. The diluent should be sterile. A suitable borate Buffer is:

$Na_2B_4O_7,10H_2O$: 3.63 g
H_3BO_3: 5.25 g
NaCl: 6.19 g
Tween: 0.0005%
Distilled Water: to 1 liter

The preferred strain of *M. vaccae* is one denoted R877R isolated from mud samples from the Lango district of Central Uganda (J. L. Stanford and R. C. Paul, Ann. Soc. belge Med, trop. 1973, 53, 141-389). The strain is a stable rough variant and belongs to the *aurum* sub-species. It can be identified as belonging to *M. vaccae* by biochemical and antigenic criteria (R. Bonicke, S. E. Jahasz., Zentr albl. Bakteriol. Parasitenkd. Infection skr. Hyg. Abt. 1, Orig., 1964, 192, 133). *M. vaccae* is believed to be closely similar antigenically to *M. leprae* (J. L. Stanford et al, British Journal of Experimental Pathology, 1975, 56, 579).

The strain denoted R877R has been deposited at the National Collection of Type Cultures (NCTC) Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, United Kingdom on Feb. 13th, 1984 under the number NCTC 11659.

For the preparation of the immunotherapeutic agent, the microorganism *M. vaccae* may be grown on a suitable solid medium. A modified Sauton's liquid medium is preferred (S. V. Boyden and E. Sorkin., J. Immunol. 1955, 75, 15) solidified with agar. Preferably the solid medium contains 1.3% agar. The medium inoculated with the microorganisms is incubated to enable growth of the microorganisms to take place, generally at 32° C. for 10 days. The organisms are harvested, then weighed and suspended in a diluent. The diluent may be saline but it preferably also contains a surfactant such as Tween 80. 1 part Tween 80 is preferably used in 300 parts saline. The suspension is diluted with the saline/-Tween 80 diluent to give 100 mg of microorganism/ml. For further dilution, borate buffered saline is preferably used to that the suspension contains 10 mg of microorganisms/ml of diluent. The suspension may then be dispensed into 5 ml multidose vials. The microorganisms in the vials are killed using irradiation e.g. from ^{60}Cobalt at a dose of 2.5 megarads, or by any other means, for example by heat.

The immunotherapeutic agent is in general administered by injection in a volume in the range 0.1–0.2 ml given intradermally. A single dosage may contain from 10^7 to 10^{10} killed *M. vaccae* microorganisms. It is preferred to administer to patients suffering from mycobacterial disease a single dose containing 10^7 to 10^{10} killed *M. vaccae*. However, the dose may be repeated depending on the condition of the patient.

The immunotherapeutic agent is preferably administered as an adjunct to chemotherapy, and normally 1 to 3 months after starting effective chemotherapy, e.g. with one of the chemotherapeutic agents mentioned above. Thus its effect is designed to be maximal after the majority of bacilli in the lesions, i.e. the metabolically active bacilli, have been killed and the load of bacterial antigenic material has begun to decline.

The invention therefore includes within its scope a method of treating mycobacterial disease, e.g. tuberculosis or leprosy, which comprises administering to a subject suffering therefrom antigenic material derived from *Mycobacterium vaccae* in an amount sufficient to provoke an immune response effective against metabolically inactive cells of mycobacteria.

The immunotherapeutic agent is believed to have two modes of action. It presents the "protective" common mycobacterial antigens to advantage and contains immune suppressor determinants active in regulating disadvantageous immune mechanisms (P. M. Nye et al, Leprosy Review, 1983, 54, 9). As a result of its action, "persister" bacilli are recognised by the immune system by their content of common mycobacterial antigens and effective immune mechanisms are directed against them, in the absence of the tissue necrotic form of immunity usually present in mycobacterial disease (G. A. W. Rook & J. L. Stanford, Parasite Immunology, 1971, 1, 111). Thus "persisters" are eradicated by the action of the body defence mechanism and the period of chemotherapy required is drastically shortened. This dramatically reduces treatment costs, and the problem of patient non-compliance with chemotherapy.

It may be advantageous and is within the scope of the invention to use more than one strain of *M. vaccae*, and/or to include in the immunotherapeutic agent other mycobacterial antigens.

The immunotherapeutic agent may also contain BCG (Bacillus Calmette-Guerin) vaccine, in particular the freeze-dried form of the vaccine, to promote its effect.

The inmunotherapeutic agent can contain further ingredients such as adjuvants, preservatives, stabilisers etc. It may be supplied in sterile injectable liquid form or in sterile freeze-dried form which is reconstituted prior to use.

The following Example illustrates the invention.

EXAMPLE

M. vaccae is grown on a solid medium comprising modified Sauton's medium solidified with 1.3% agar. The medium is inoculated with the microorganism and incubated for 10 days at 32° C. to enable growth of the microorganism to take place. The microorganisms are then harvested and weighed and suspended in diluent (1 part Tween 80 in 300 parts saline) to give 100 mg of microorganisms/ml of diluent. The suspension is then further diluted with saline to give a suspension containing 10 mg of microorganisms/ml of diluent and dispensed into 5 ml multidose vials. The vials containing the live microorganism are then subjected to radiation from ^{60}Cobalt at a dose of 2.5 megarads to kill the microorganisms and give the immunotherapeutic agent of the invention, which may (if desired) be further diluted for use.

This immunotherapeutic agent may be administered by intradermal injection in the manner already described.

What is claimed is:

1. An immunotherapeutic agent consisting essentially of killed cells of *Mycobacterium vaccae*.

2. An immunotherapeutic agent according to claim 1 comprising cells of *M. vaccae* which have been killed by irradiation.

3. An immunotherapeutic agent according to claim 1 in sterile injectable liquid form or in sterile freeze-dried form.

4. An immunotherpeutic agent according to claim 1 derived from *M. vaccae* NCTC 11659.

5. An immunotherapeutic agent according to claim 1 in the form of a single dosage unit containing 10^7 to 10^{10} killed cells of *M. vaccae*.

6. An immunotherapeutic agent according to claim 1 which also comprises BCG vaccine.

7. An immunotherapeutic agent according claim 5 which also comprises BCG vaccine.

8. An immunotherapeutic agent according to claim 1 which also comprises one or more additional ingredients selected from adjuvants, preservatives, and stabilizers.

9. Method of treating mycobacterial disease which comprises administering to a subject suffering therefrom, in association with chemotherapy, killed cells of *Mycobacterium vaccae* in an amount sufficient to provoke an immune response effective against mycobacteria that survive the chemotherapy.

10. Method according to claim 9 in which the mycobacterial disease is tuberculosis or leprosy and the mycobacteria are *Mycobacterium tuberculosis* or *M. leprae* respectively.

11. Method according to claim 9 in which the antigenic material comprises cells of *M. vaccae* NCTC11659 which have been killed by irradiation.

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Display Settings: Abstract

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Mycobacterium vaccae immunotherapy for treating tuberculosis.

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Update of:

Cochrane Database Syst Rev. 2001;(1):CD001166.

BACKGROUND: Some authorities have advocated Mycobacterium vaccae immunotherapy for treating tuberculosis and other infections caused by mycobacteria. OBJECTIVES: To assess the effects of Mycobacterium vaccae as an adjunct to chemotherapy for treating tuberculosis. SEARCH STRATEGY: We searched the Cochrane Infectious Diseases Group trials register (September 2002), the Cochrane Controlled Trials register (Issue 3, 2002), MEDLINE (1966 to October 2002), EMBASE (1980 to September 2002), and reference lists of articles. We also contacted organisations and individuals working in the field. SELECTION CRITERIA: Randomised and quasi-randomised trials using whole, killed Mycobacterium vaccae for patients with tuberculosis. DATA COLLECTION AND ANALYSIS: One reviewer assessed trial quality and extracted data. MAIN RESULTS: Seven trials met the inclusion criteria. There was no effect on mortality (4 trials, OR 1.09, 95% CI 0.79 to 1.49). No consistent effect on sputum negativity or sputum culture was shown. Most immunotherapy recipients experienced local adverse reactions (2 trials, OR 18.2, 95% CI 9 to 37), some of which progressed to ulceration and scarring. REVIEWER'S CONCLUSIONS: Mycobacterium vaccae does not benefit patients with tuberculosis.

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